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Soybean Seed Production and Nitrogen Nutrition

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1. Introduction

The world population is consistently increasing, and it is over 7 billion in 2012, while the land area for agricultural use is limited. Therefore, the increase in crop production per area is very important. Soybean (*Glycine max* (L.) Merr.) originates from East Asia, and soybean seed is one of the most important protein sources for human and livestock all over the world. Annual production of soybean (262 M (million) t in 2010) is the fourth of the major grain crops, after maize (844 M t), paddy rice (672 M t) and wheat (650 M t) [1]. In the whole world, over 85% of soybean is used for oil and the residue is used for animal feed. Annual soybean seed production has been steadily increasing for recent decades (91 M t in 1980, 109 M t in 1990, 161 M t in 2000, 262 M t in 2010) [1]. The cultivation area of soybean is 102 M ha in 2010. Major soybean production countries (annual production in 2010) are USA (90.6 M t), Brazil (68.5 M t), Argentina (52.7 M t), China (15.1 M t), and India (9.8 M t) in this sequence. Soybean production in Japan in 2010 was only 223,000t and it accounted for 5% of the total consumption in Japan. The world average seed yield is 2.56 t ha^{-1} in 2010, and is higher in the USA (2.92 t ha^{-1}), Brazil (2.94 t ha^{-1}), and Argentina (2.90 t ha^{-1}) compared with China (1.77 t ha^{-1}), Japan (1.62 t ha^{-1}) and India (1.07 t ha^{-1}) and other countries.

The nutrient composition of soybean seeds (per 100 g) produced in Japan is as follows [2]; energy 417 kcal (1,745 kJ), water 12.5g, protein 35.3g, lipids 19.0g, carbohydrate 28.2g, minerals 5g. The composition is quite different from the other grain crop seeds, such as "Paddy rice"; energy 350 kcal (1,464 kJ), water 15.5g, protein 6.8g, lipids 2.7g, carbohydrate 73.8g, minerals 1.2g, "Wheat"; energy 337 kcal (1,410 kJ), water 12.5g, protein 10.6g, lipids 3.1g, carbohydrate 72.2g, minerals 1.6g, and "Corn"; energy 350 kcal (1,464 kJ), water 14.5g, protein 8.6g, lipids 5.0g, carbohydrate 70.6g, minerals 1.3g. The protein concentration in soy-

bean seeds is very high about 4-5 times higher than that of rice, wheat and corn, but carbohydrate concentration is lower.

The storage protein of soybean seeds mainly consists of glycinin and β-conglycinin. The β-conglycinin is comprised of three subunits, designated as α', α, and β-subunits. The β-subunit of β-conglycinin is especially low in sulfur amino acids, containing only one cysteine and no methionine residue in its mature form. Soybean seeds contain a large amount of lipids (20%), and about 90% is unsaturated fatty acid (linoleic acid 51-57%, oleic acid 32-36% and linolenic acid 2-10%) and 10% is saturated fatty acid (palmitic acid 4-7%, stearic acid 4-7%) [3]. Linoleic acid and linolenic acid are essential lipids, which cannot be synthesized by ourselves. Although soybean seeds contain about 28% carbohydrates, most of them are structural carbon like cell walls and oligosaccharides (sucrose 5%, stachyose 4%, raffinose 1%). Starch is tentatively accumulated in young immature soybean seed, however, it decreases and converts to lipid and protein at maturity. Soybean seeds contain relatively a high amount of minerals (5%) compared with cereal seeds (about 1%). Soybean seeds contain abundant potassium (1,900 mg), calcium (240 mg), magnesium (220 mg), phosphorous (220 mg), iron (9.4 mg), zinc (3.2 mg) per 100g seeds. Soybean seeds contain vitamins, both lipid soluble vitamins (Vitamin E (1.8 mg)) and water soluble vitamins (V B_1 (0.83 mg) and V B_2 (0.30 mg)). Soybean seeds contain isoflavonoids, daidzein and genistein. These isoflavonoids are expected to play a role as a female hormone or to decrease fat in blood.
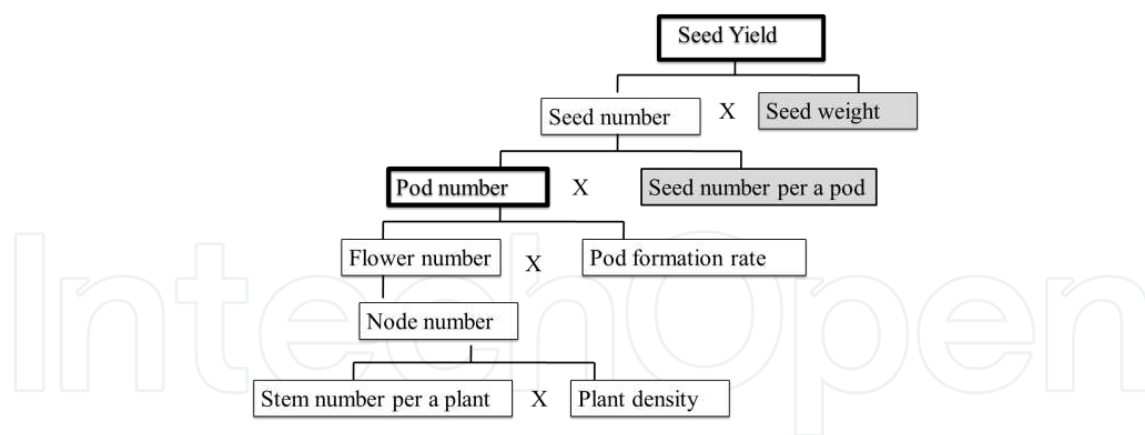
2. Soybean seed yield

2.1. Potential of soybean yield

The world average soybean yield has been increasing by about 60% for 30 years from 1980 (1.6 t ha^{-1}) to 2010 (2.6 t ha^{-1}). The highest yield of soybean in Japan was recorded at 7.8 t ha^{-1}, and soybean seed yield can reach 4-6 t ha^{-1} with well-managed fields under good climatic and soil conditions [4]. Recently, an amazing high soybean seed yield over 10 t ha^{-1} was recorded in 2008 and 2010 by a farmer, Mr. Kip Cullers in Missouri, USA [5]. Therefore, the potential productivity of soybean should be much higher than we have thought.

Figure 1 shows the yield components of soybean. Soybean "seed yield" is calculated by multiplying the "seed number" per area and one average "seed weight". Seed number per area is calculated by the "pod number" and the average "seed number per pod". The pod number is decided by "flower number" and the "pod formation rate". The flower number depends on the "node number". The node number per area is decided by "stem number per plant" and "planting density".

Farmers can control the planting density. Planting density is an important factor for soybean growth and seed yield, although the planting density is not directly proportional to the dry matter production and seed yield. When planting density is high, the branching of each plant is depressed and the number of the lateral stems decrease. In addition, under excess planting density the competition for photosynthesis and nutrient absorption among plants become severe and the stems grow tall and thin and plants are proneto lodge.

Figure 1. Yield components in soybean cultivation.

Generally, soybean seed yield depends mostly on pod number per area. Generally over 50% of soybean flowers and young pods abort and they don't make mature pods and seeds. Under bad weather and growing conditions, the percentage of flower and pod abortion increases. The average number of seeds in a pod is relatively constant, although the seed number per pod differs from 1 to 4 in soybean. Average seed weight is affected by growing conditions in late growth stages.
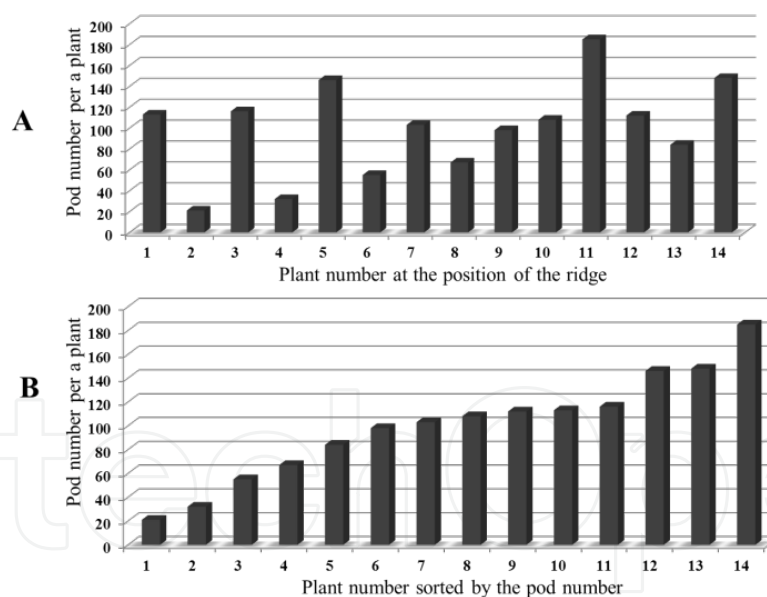

Figure 2. Pod number per a plant grown in the same row in Nagakura field in 2011. A: The number shows the original position in the row. B: The number of the plant is sorted from low to high pod number.

The low average yield compared with potential productivity (10 t ha⁻¹) may be due to several factors that interfere with maximum growth. First, soybean plants are very susceptible to physical, chemical and biological conditions of the soil as well as climatic conditions. Figure 2 shows the pod number per plant of soybean plants grown in a row. Figure 2A shows the data of the plants at the original position of the row, and Figure 2B shows data are sorted

from low to high pod number. In this row, the highest pod number was about 180 and the lowest was only 18, and the average pod number was about 100 pods per a plant. As shown in Figure 2A, a plant with many pods tended to neighbor to a plant with low pod number. This may be mainly due to competition for solar radiation, and the plant growth is easily depressed by shading of neighbor bigger plant.

Another example of adaptation of soybean plants to environmental conditions is shown in Figure 3 and 4. When soybean plants are planted in a small pot, the growth was inferior (Figure 3) and the "dwarf" soybean formed only 3-5 pods with normal seeds. Figure 4 shows an example of "giant" soybean cultivated with a low planting density at 2 plants m^{-2} [6]. This plant had a very thick basal stem (25 mm diameter), and the dry weight per a plant was 572g (leaves 100g, stems 204g, pods 220g, roots 41g, and nodules 7g). It had 1,874 nodules on the roots. The plant had 17 lateral branches, 178 stem nodes, 600 pods and 1,687 seeds as shown in Figure 5.
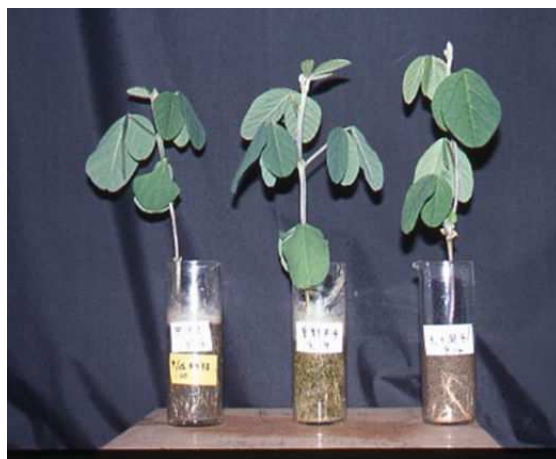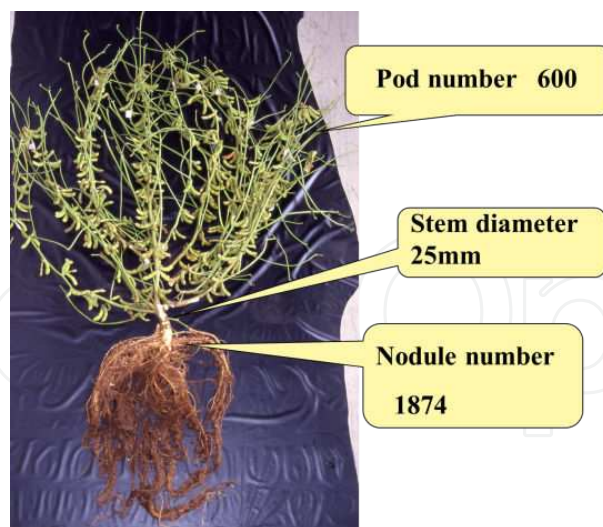

Figure 3. Soybean plants grown in a small flask.

Figure 4. Soybean plants (cv. Williams) cultivated at the density of 2 plants m^{-2} in Ikarashi sandy dune field in 2000. [6]

Figure 5. Characteristics of soybean plant (cv. Williams) cultivated at the density of 2 plants m^{-2} in Ikarashi sandy dune field in 2000. [6]

2.2. Characteristics of soybean growth

Optimal planting date for a cultivar in the growing area is very important to get good growth and seed yield. When planting is delayed only a few weeks, the stem length and plant dry matter accumulation at R1 stage may decrease by half. Optimum planting density is also important. In Niigata, the planting density for cultivar "Enrei" is 89,000 plants ha^{-1} by a single seed planting, which means one seed is planted in each seeding spot, with 75 cm row spacing and 15 cm planting distance in a row. When the germination rate of the seeds is not good, the planting density will decrease. Therefore, multiple seeding is sometimes carried out to avoid subnormal plant population. However, the total number of nodes, pods and seeds per area are almost the same between single-seeded and multi-seeded planting.

Figure 6 shows the stage description for the vegetative and reproductive growth of soybean proposed by Fehr and Caviness [8]. Soybean seeds germinate in a few days and emergence of seedling occurs about 7-10 days after sowing (PE stage), if soil moisture and temperature are optimum. After cotyledon leaves appear, a pair of unifoliolate leaves unroll (VC stage). During this stage, the storage compounds in the cotyledons support the nutrition for root and shoot growth. Then trifoliolate leaves appear one by one at V1 (the first trifoliolate leaf), V2 (the second trifoliolate leaf) and so on (Vn stage), and the shoot and roots grow during the vegetative stage.

The reproductive growth starts from beginning bloom (R1 stage). Soybean is short-day plant, and they bloom when the day length become shorter than 14 hrs, although it depends on the varieties and planting date. Bloom period lasts for 15-50 days from the beginning to the end of bloom. Full bloom is described as R2 stage. Pod initiation starts (R3 stage) about one month after the R1 stage. Then about one month after R3 stage, the seed begins to enlarge (R5 stage) to full seed (R6 stage). By one month after R5, the soybean plant starts to become mature (R7 stage). At 2-3 weeks after the R7 stage, the plant dries down to harvest

maturity (R8 stage). Vegetative growth continues after R1 in both determinate and indeterminate soybeans. Vegetative growth of stems and leaves does not stop until about the R5 stage.

Vegetative (V) soybean growth stages

PE: Plant emergence (depends on temperature and moisture).
VC: Unifoliolate leaves unrolled in addition to cotyledons. One node.
V1: One unrolled trifoliolate leaf. Two nodes.
V2: Two unrolled trifoliolate leaves. Three nodes.
Vn: (n) number of trifoliolate leaves unrolled; (n) + 1 number of nodes

Reproductive (R) soybean growth stages

R1: Beginning bloom. At least one flower is present on the main stem.
R2: Full bloom. Flowers are found on any of the top two nodes.
R3: Beginning pod. Pods are 3/16 inch (4.8 mm) long on one of the top four nodes.
R4: Full pod. Pods are 3/4 inch (19 mm) long on one of the top four nodes.
R5: Beginning seed. Seeds are 1/8 inch inch (3.2 mm) long on one of the top four nodes.
R6: Full seed. Pods are completely filled by seeds on one of the top four nodes.
R7: Beginning maturity. One mature pod found on the plant.
R8: Full maturity. 95% pods have reached mature pod color.

Figure 6. Stage description for the vegetative and reproductive growth of soybean. (from Clemson Cooperative Extension, Home Page)

Root growth starts with a seminal root, which becomes a primary root. The secondary roots are formed from the primary root. The first root nodules are formed on the basal part of the primary roots, and they become visible at about 10 days after planting. They start to fix nitrogen (N_2) at about 15-20 days after planting when the diameter reaches about 2 mm [9]. In the later stage, the nodules formed at the basal part of primary roots degrade, and a large number of new nodules form on the lateral roots near the soil surface, and they play an important role for supplying N during the pod filling stage.

2.3. Factors affecting soybean yield

Soybean plants are very susceptible to environmental conditions, such as climatic conditions (solar radiation, day length, temperature, rain fall etc), soil conditions (drought, excess water, pH, soil fertility, mineral nutrition, etc). Secondly, soybean seed yield often severely declines with pests, such as insects, weeds, diseases, and nematodes. Third, nitrogen fixation by the root nodules (Figure 7) with the soil microorganism bradyrhizobia is very important for soybean production [10,11], however, it is difficult to obtain the optimum condition of nodulation and nitrogen fixation. The nodule formation and nitrogen fixation is sensitive to the external factors such as climate, soil properties, pests etc, and internal factors such as competition among plants or competition among organs, pods, leaves, roots and nodules. Therefore, many stress conditions, such as drought stress, decrease in oxygen supply, a high or low pH, nutrient imbalance etc., may depress nodule formation and nitrogen fixation activity. In addition, low population of compatible bradyrhizobia or the dominance of inefficient strains of indigenous bradyrhizo-

bia in the field may decrease nitrogen fixation activity. The inoculation of efficient strains of bra-dyrhizobia may promote soybean growth and seed yield.

Figure 7. A photograph of the nodulated roots of soybean plant (cv. Williams) inoculated with *Bradyrhizobium japonicum* USDA110 and cultivated in a glass bottle with culture solution.

In Japan, over 80% of soybean cultivation is carried out in rotated paddy fields by block rotation with rice, because rice has been over produced in Japan. For example, one block of rice field is drained and soybean cultivation continued there for 3 years. Then this field is returned to rice cultivation for the next 3 years. When drainage of water is good and the ground-water level is maintained at lower than 30cm, soybean cultivation will be successful. However, bad drainage of water like in heavy clay soil near Niigata will depress root and nodule development. Therefore, soybean growth and seed yield is very poor. Hosokawa developed a new method of a raised planting bed cultivation by changing the blades of a reverse rotary tiller. Soybean plants grow very well in a raised bed due to efficient drainage of water especially after heavy rain fall. Nagumo et al. reported that a higher seed yield has been obtained by raised bed tillage with sigmoidal releasing-type coated urea fertilizer in the rotated paddy field under poor drainage conditions. Respiration of nodules and roots is severely depressed by excess water in soil due to oxygen deficiency. Soybean plant can be cultivated in water culture (Figure 7) when aeration is good. However, under water logging conditions, oxygen deficiency in soil occurs, because diffusion of oxygen through water is very slow compared with gas diffusion through soil, and soil microorganisms respire O_2 actively under high temperature conditions. It is known that soybean nodule respiration is about 4 times higher than that in roots in order to support nitrogen fixation activity and nitrogen assimilation to ureides. Therefore, low oxygen supply is fatal for nodules.

2.4. Nitrogen assimilation and seed yield

Soybeanplants assimilate a large amount of nitrogen during both vegetative and reproductive stages, and the total amount of N assimilated in a plant is highly correlated with the soybean seed yield. One t of soybean seed requires about 70-90 kg N, which is about four

times more than in the case of rice [13]. Soybean plants assimilate the N from three sources, 1) N derived from symbiotic N_2 fixation by root nodules (Ndfa), 2) absorbed N from soil mineralized N (Ndfs), and 3) N derived from fertilizer when applied (Ndff) (Figure 8). For the maximum seed yield of soybean, it is necessary to use both N_2 fixation and absorbed N from roots [14-15]. When only N_2 fixation is available to the plant vigorous vegetative growth does not occur, which results in reduced seed yield. On the other hand, a heavy supply of N often depresses nodule development and N_2 fixation activity and induces nodule senescence, which also results in reduced seed yield. In addition, a heavy supply of N from fertilizer or from the soil causes luxuriant shoot growth, which result in lodging and poor pod formation. Therefore, for soybean cultivation no nitrogen fertilizer is applied or only a small amount of N fertilizer is applied as a "starter N" to promote the initial growth.
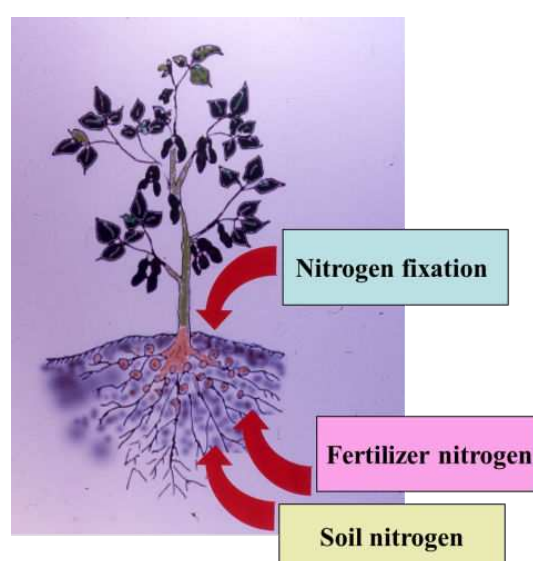
Figure 8. Three sources of nitrogen for soybean plants, nitrogen fixed in root nodules, nitrogen absorbed by roots from fertilizer and from soil nitrogen.

In Niigata fields, about 60-75% of N assimilation in soybean was estimated to derive from N_2 fixation [16,17]. Figure 9 shows the growth of cultivar "Enrei" and the non-nodulated isogenic line "En1282" planted in the same Nagakura field. It is obvious that non-nodulated soybean grew very poor with pale leaf color due to N deficiency by the lack of nitrogen fixation. It is said that the legume nitrogen fixation is variable, but it is a valuable process in world agriculture [18-20].

3. Characteristics of nitrogen nutrition of soybean

3.1. Characteristics of nitrogen nutrition related to seed yield

Soybean seed contains an extraordinary high concentration of protein about 35-40% based on the seed weight. Many field researches showed the soybean seed yield is proportional to

the total assimilated N in plants. Figure 10 shows the relationship between total amounts of N in soybean shoot at the R7 stage and seed yield in rotated paddy field in Nagakura from 1989-1991 [21]. The seed yield exhibited a linear correlation (r=0.855) with the amount of nitrogen accumulation.


Figure 9. Comparison of the growth of nodulating soybean cv. "Enrei" (left) and the non-nodulated mutant "En1282" (right) cultivated in Nagakura field.
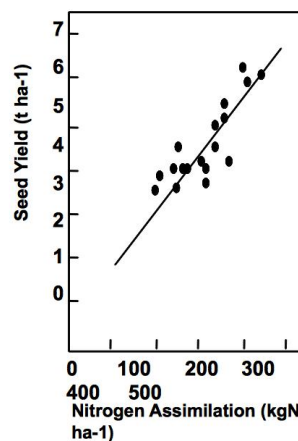
Figure 10. Relationship between amount of nitrogen accumulated in soybean shoot at R7 stage and seed yield of soybean cv. "Enrei". In Nagakura field with various fertilizer treatments.[21]

The protein concentration in soybean seeds is about 4 times higher than cereal seeds such as rice grain (Figure 11) [12]. Due to a high concentration of seed protein, 1t of soybean seed production requires about 70-90 kg of N, while 1t of rice grain requires only 20 kg of N. Soy-

bean plants assimilate about 20 % of total N until initial flowering stage (R1 stage), and 80% of N during the reproductive stage. On the other hand, rice assimilates about 80% of N until flowering. Therefore, the continuous assimilation of nitrogen after initial flowering stage is essential for good growth and high seed yield in soybean cultivation.
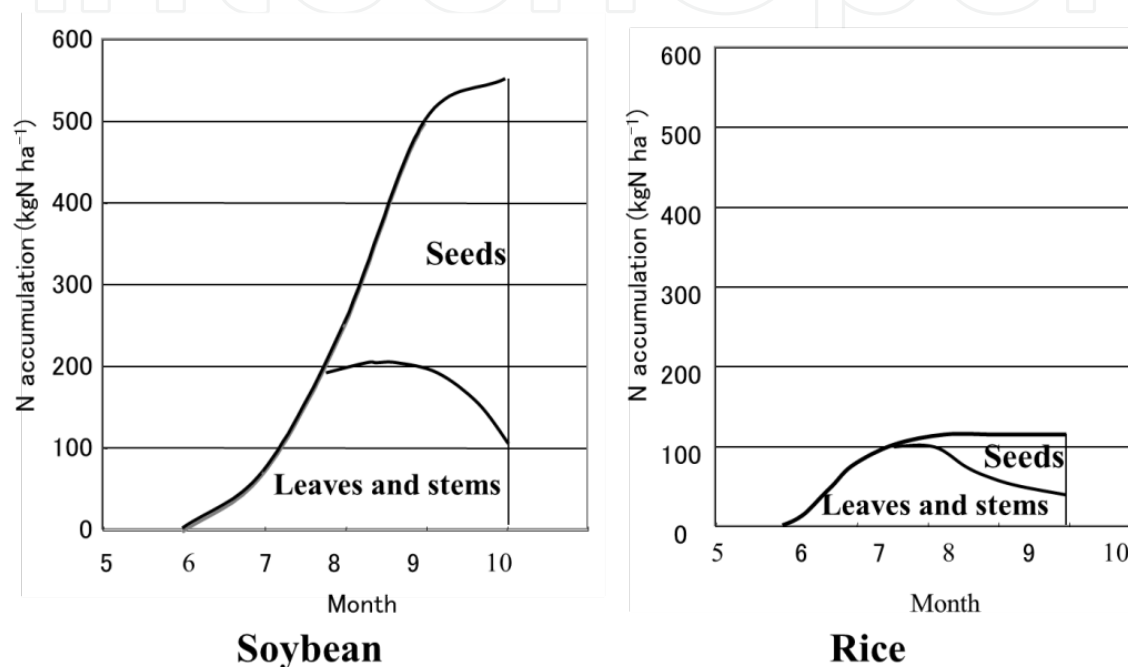

Figure 11. Comparison of the nitrogen assimilation and distribution pattern of soybean and rice.

To obtain high seed yield of soybean, good nodulation and high and long lasting nitrogen fixation activity are very important (Figure 12). Nodule formation and nodule growth are influenced by various soil conditions (water content, pH, nutrition) and climatic conditions (solar radiation, temperature, rain fall etc). Soybean can fix atmospheric N_2 by their root nodules associated with soil bacteria, bradyrhizobia. In addition, soybean can absorb inorganic nitrogen, such as nitrate and ammonia from soil or fertilizer. Usually a high yield of soybean was obtained in a field with high soil fertility. By supplying a constant but low concentration of nitrogen either from soil or organic manure, soybean growth will occur without depressing nodulation and nitrogen fixation activity. However, it is well known that a high concentration of mineral N depresses nodule formation and nitrogen fixation activity. Especially, nitrate, the most abundant inorganic nitrogen in upland fields, severely inhibits nodulation and nitrogen fixation of soybean, when nodulated roots are in direct contact with the soil solution containing nitrate [22-24].
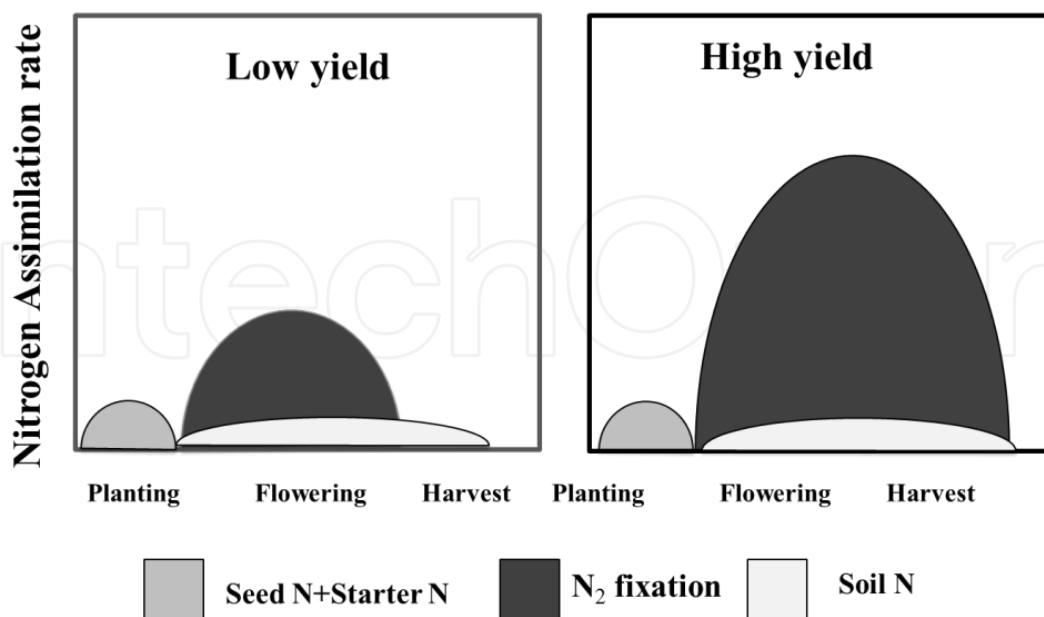
Figure 12. Comparison of the time course of nitrogen assimilation in soybean plants with a low yield (left) and a high yield (right).
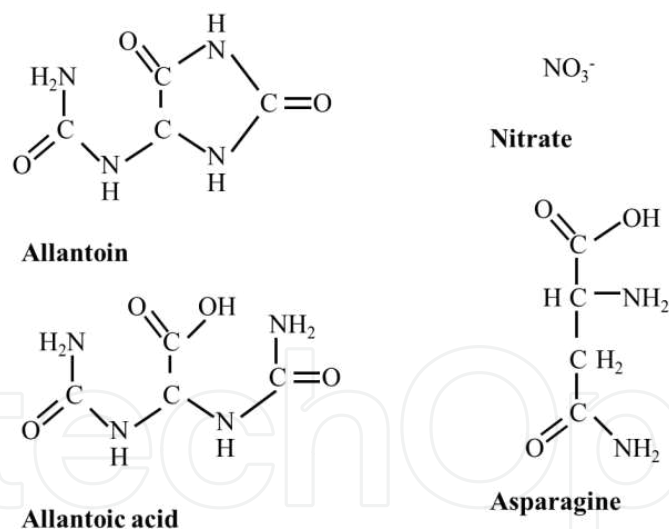

Figure 13. Chemical formula of ureides (allantoin and allantoic acid), nitrate and asparagine.

3.2. Nitrogen assimilation in nodules

Ammonia is known to be the initial product of biological nitrogen fixation by the enzyme nitrogenase [25]. After discovering an enzyme glutamate synthase (GOGAT) in *Aerobacter aerogenes* [26], it was confirmed that ammonia can be assimilated via glutamine synthetase (GS) and glutamate synthase (GOGAT) pathway in soybean nodules by ^{15}N tracer experi-

ments [27,28]. The ^{15}N assimilation was investigated in the cytosol (plant cytoplasm) and bacteroid fractions of soybean nodules [29]. The result suggested that most of the fixed N is immediately exported from the bacteroid to the plant cytosol and assimilated via GS/GOGAT pathway into various amino acids via transamination from glutamate. Ureides, allantoin and allantoic acid are synthesized from amino acids and amides in the cytosol (Figure 13,14). Kushizaki et al. discovered that nodulated soybean plants contain a large amount of ureides in their stems, while non-nodulating isolines contain much less [30]. Reviews on ureide biosynthesis in legume nodules were published [31,32].
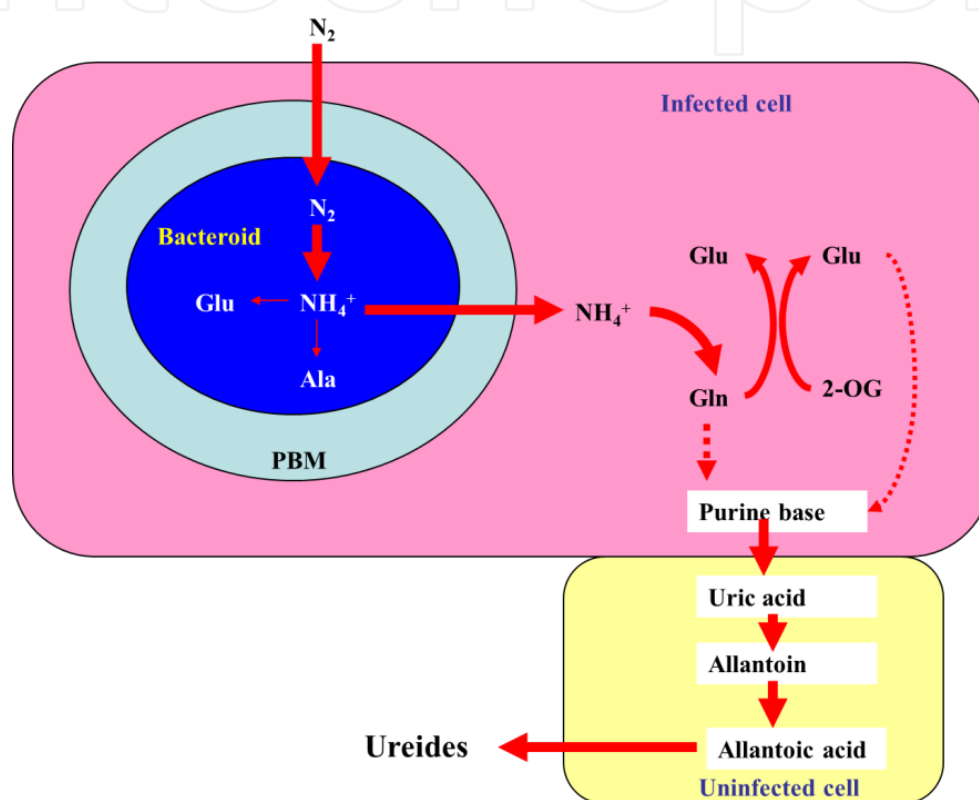

Figure 14. A model of the flow of fixed N_2 in infected cell and uninfected cell of soybean nodule.

3.3. Nitrogen absorption and assimilation in soybean roots

The outlines of absorption and metabolism of ammonium and nitrate in plant cells are shown in Figure 15. Ammonium (NH_4^+) and nitrate (NO_3^-) are major sources of inorganic nitrogen in soil. Ammonium is the most reduced form of nitrogen and nitrate is the most oxidized form. The NH_4^+ ion is absorbed through the membrane bound protein, ammonium transporter. The NO_3^- ion is absorbed through the nitrate transporter with $2H^+$ co-transport. There are two types of nitrate transporter, a high affinity nitrate transporter system (HATS) and a low affinity nitrate transporter system (LATS) [33]. The kinetics of the absorption rate versus nitrate concentration indicated the presence of only one HATS, having a Km value of 19 μ mole in soybean roots.
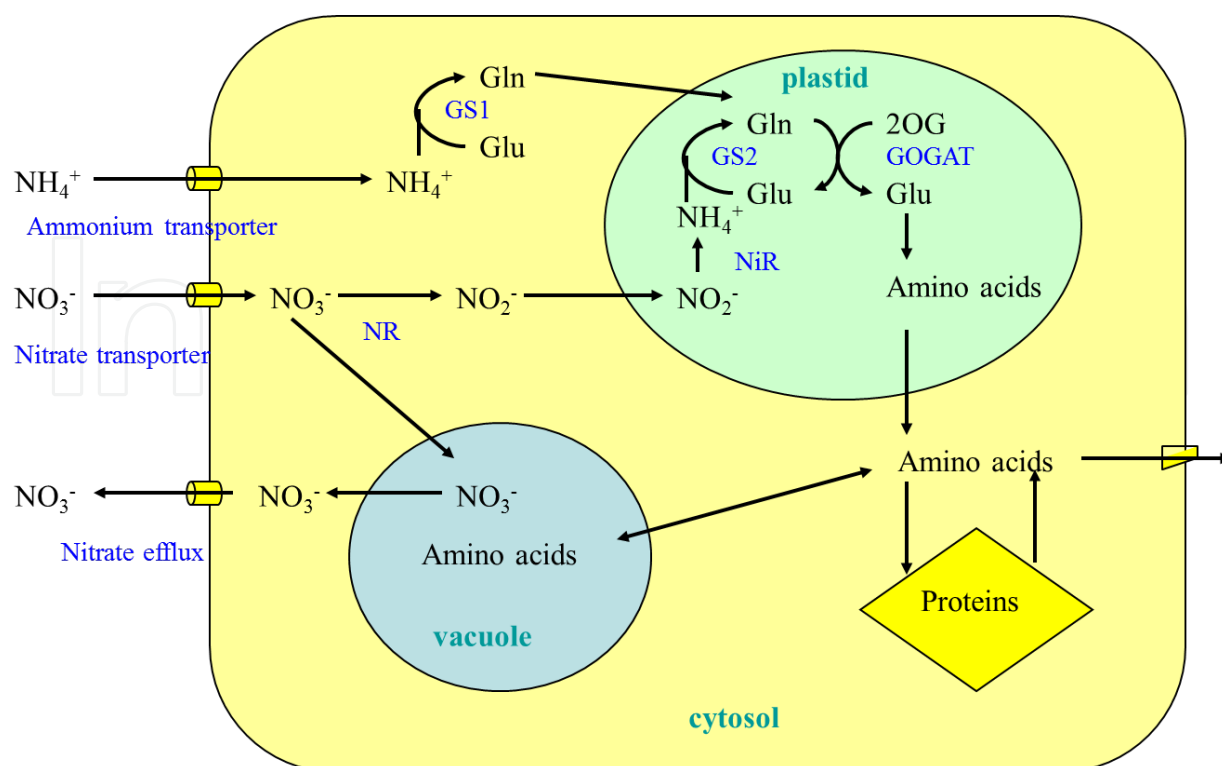
Figure 15. A model of absorption and metabolism of ammonium and nitrate in plant cell.

The diurnal rhythm in NO_3^- absorption by intact soybean plants was investigated by sampling the culture solution every 15 min [34]. The NO_3^- absorption rate was different between day (1.10 mgN L^{-1} h^{-1}) and night period (0.77 mgN L^{-1} h^{-1}), and the nitrate absorption rate at night was about 60-75% of that in the daytime. The temporary interruption of NO_3^- absorption was observed twice a day at dawn and dusk. The changes in NO_3^- absorption rate were not controlled by the shoots, because the rhythm continued under the extended dark period or by cutting the shoots [34]. When the roots were put in the water bath at a constant temperature of 30°C, the rhythm of NO_3^- absorption disappeared. This suggests that the nitrate absorption rate of soybean roots may be controlled by monitoring temperature changes by the root.

Some parts of the NO_3^- absorbed in the root cell is reduced to nitrite (NO_2^-) by nitrate reductase (NR) in the cytosol, and NO_2^- is reduced to ammonia by nitrite reductase (NiR) in the plastids. Then amino acids are formed followed by the assimilation via GS/GOGAT pathway. When a high concentration of NO_3^- is supplied, a part of NO_3^- is temporarily stored in the vacuoles of cortex cells in roots. Some parts of NO_3^- are transported cell to cell via the symplast pathway and effluxed in the stele and transported via the xylem with the transpiration stream in the form of NO_3^-. Plant NR requires NADH or NAD(P)H which means both NADH and NADPH act as electron donors. In soybean, there are two types of NAD(P)H-NR and one type of NADH-NR [15].

After adding $^{15}NO_3^-$ in the solution, the ^{15}N concentration of asparagine increased markedly, indicating that asparagine is a major assimilatory compound of NO_3^- in soybean roots. Ni-

trogen assimilation and transport of plants supplied with $^{15}NO_3^-$ was investigated by analyzing xylem sap collected from decapitated soybean plants [35]. Some part of the NO_3^- absorbed in the roots was immediately exported to the shoots, whereas another parts of the NO_3^- was temporarily stored in the vacuoles of root cells and then gradually released to the xylem. On the other hand, some other parts of the NO_3^- was reduced and assimilated in the roots and synthesized into asparagine.
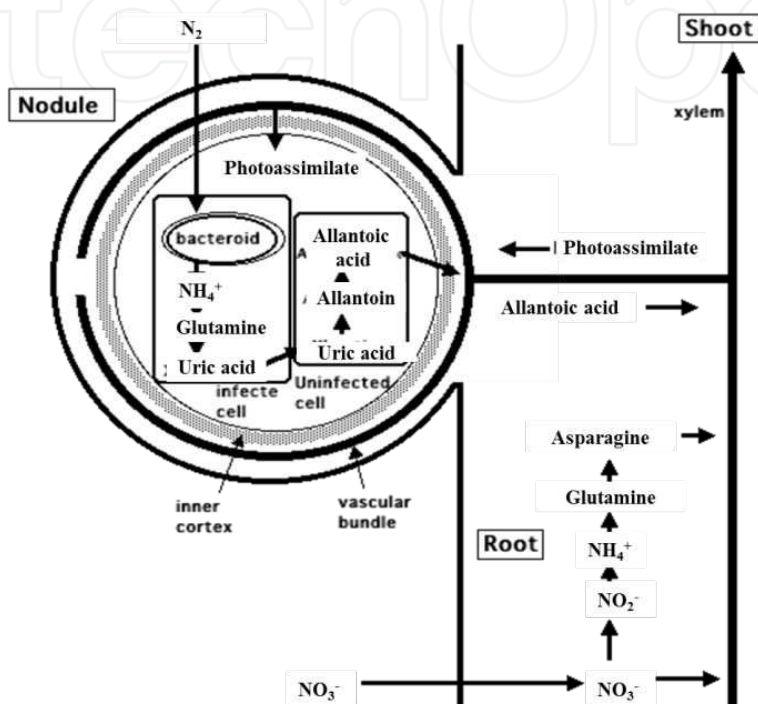
Figure 16. Comparison of the fate of fixed N_2 in nodules and absorbed nitrate in roots.

3.4. Comparison of the fate of fixed N_2 and absorbed NO_3^- in soybean plant

The labeling patterns of ureides and amino acids were compared from the data of $^{15}N_2$ and $^{15}NO_3^-$ feedings [36]. The result proved that the ureides in stems are mainly derived from fixed N_2, and only a small amount of ureides is synthesized in the root. Figure 16 shows the metabolic pathways and transport of N derived from N_2 fixation and NO_3^- absorption in soybean plants.

3.5. Effect of combined nitrogen on nodule growth and nitrogen fixation activity

It is well known that a heavy supply of nitrogen fertilizer causes the inhibition of nodulation and nitrogen fixation. The inhibitory effect of combined nitrogen depends on the forms, concentration, application site, and soybean growth stage. The inhibitory effect of nitrate is stronger than urea or ammonium. Direct and indirect effects of nitrate have been known. Direct or local effect of nitrate is the effect of nitrate in direct contact with the nodulated part of roots. When nitrate was supplied to the hydroponically grown soybean roots, the nitrate in-

hibition on nodule growth and nitrogen fixation was shown to be rapid and reversible [22-24]. On the other hand, indirect or systemic effect of nitrate, which means the effect of nitrate absorbed from distant part of the roots, depended on the site, duration and concentrations of nitrate supply [37,38]. The indirect effect of nitrate was investigated by a two-layered pot system separating the upper roots and lower roots. A continuous high concentration of nitrate (5mM) supply in the lower roots depressed the nodulation and nitrogen fixation of the upper roots. However, a continuous supply of a low concentration of nitrate (1 mM) resulted in the promotion of nodulation and nitrogen fixation activity in the upper roots [38].

3.6. Nitrogen metabolism in soybean leaves

Plant leaves are the important organ for nitrogen metabolism, in addition to photosynthetic activity. The N absorbed from roots or fixed in root nodules is transported via the xylem in stems and petioles, and the N in leaves is translocated to the sink organs such as pods and seeds via the phloem. The flow of N in leaves was investigated by petiole girdling and $^{15}N_2$ or $^{15}NO_3^-$ treatment [39]. By petiole girdling treatment, the accumulation of amino acids (x 2.5) especially asparagine (x 8.8) in leaf blades was observed, indicating that these compounds are the transport forms from leaves to sink organ via the phloem. However, nitrate and ureides are not accumulated in girdled leaves compared with intact leaves, suggesting that nitrate and ureides are not transported from leaves to sinks via the phloem (Figure 17). There are two different ureide degradation pathways in soybean leaves, either by allantoate amidinohyfrolase or by allantoate amidohydrolase [40].
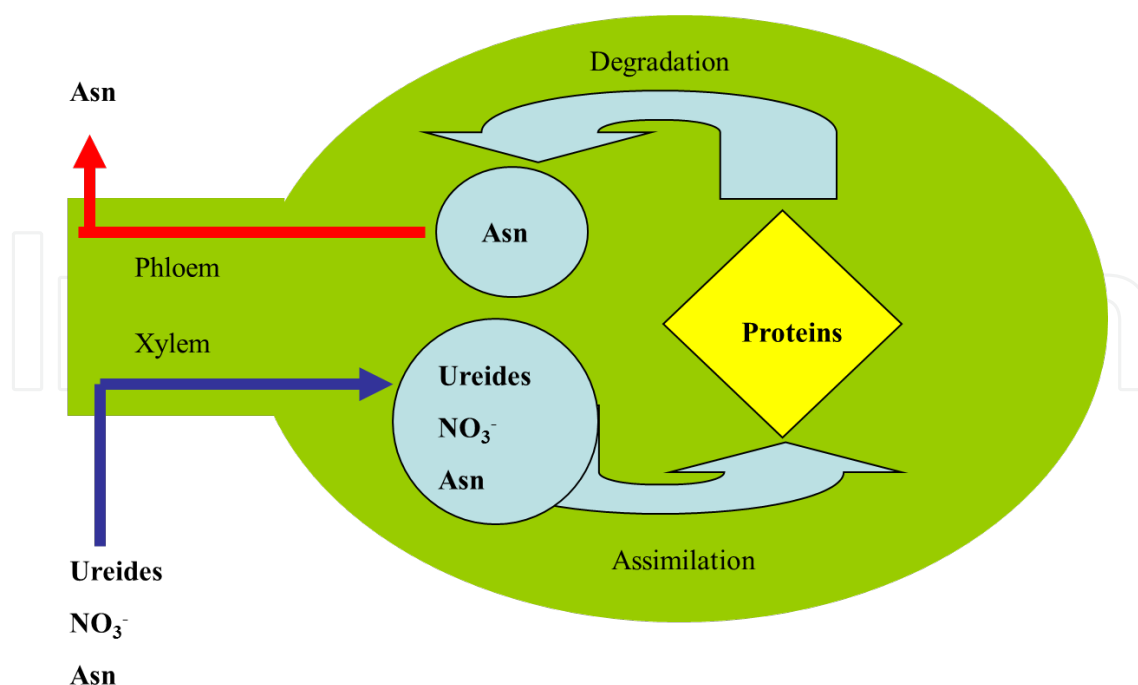
Figure 17. A model of N flow in a soybean leaf.

A comparative study on the nitrogen metabolism and transport was done at the pod filling stage by $^{15}N_2$ or $^{15}NO_3^-$ treatment [41]. Based on the results obtained, we proposed a model of N flow derived from N_2 and NO_3^- in soybean plants as shown in Figure 18 [42]. The N derived from N_2 fixed by the root nodules is rapidly assimilated into ureides (allantoin and allantoate), and some ureides are directly transported to pods and used for seed development. Ureides are also used for leaf protein synthesis, but the contribution is relatively lower than N derived from NO_3^- absorbed from the roots. On the other hand, some part of NO_3^- absorbed from the roots is immediately reduced in the roots, and transported in the form of amino acids, especially asparagine. Another part of NO_3^- is transported to the leaf blades via transpiration, and assimilated into leaf protein. The remobilization of storage protein in leaves and roots may be a major source for seed N source in the case of NO_3^- nutrition.
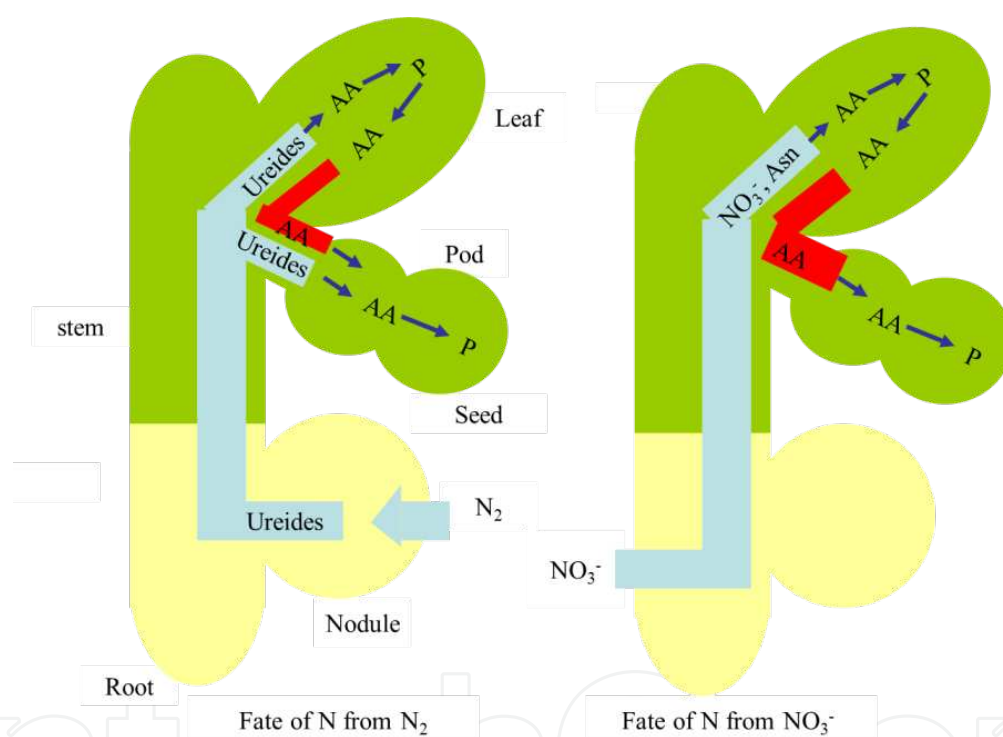

Figure 18. A model of N flow in soybean plant originated from N_2 (left) and NO_3^- (Right). AA: amino acids, P: protein

3.6. Assimilation of nitrogen in pods and seeds

Figure 19 shows the outline of the N flow in soybean pod and seed (cotyledon). Ureides are transported from the root nodules via the xylem and are accumulated in the pod. Allantoin and allantoic acids are metabolized into amino acids in the pod or seed coat and excreted to the inside of seed coat. Asparagine from roots via the xylem or from leaves via phloem is also metabolized to amino acids and then transported into the apoplast space between the seed coat and cotyledon. The cotyledon cells absorb amino acids from the apoplast and they synthesize storage proteins and accumulate them into protein bodies.

Rainbird et al. reported that glutamine is the principal N supply to the cotyledon, contributing 55% of the embryo nitrogen requirement, and 20% comes from asparagine, with negligible amounts from ureides, allantoin and allantoic acid. Haga and Sodek also reported that glutamine was the most efficient source in terms of protein accumulation in the cultured soybean cotyledons, while asparagine was less efficient and allantoin was a poor source of nitrogen. Ohtake et al. reported that the rapid N transport to pods and seeds in N-deficient soybean plants were faster compared with N-sufficient plants.
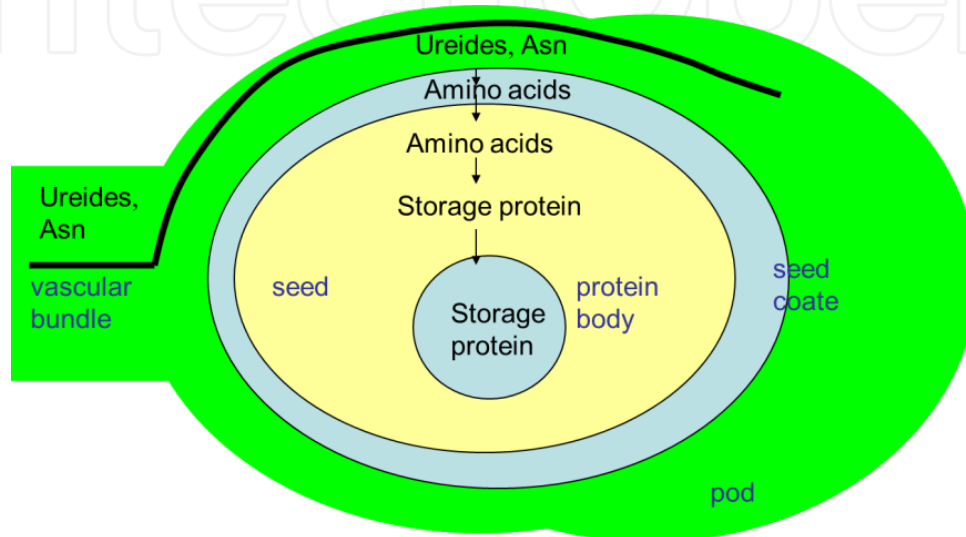
Figure 19. The model of nitrogen assimilation in soybean pod and seed.

The storage protein of soybean seeds mainly consists of glycinin and β-conglycinin. β-conglycinin is comprised of three subunits, designated as α', α, and β−subunits [46]. We happened to discover the lack of β-subunit of β-conglycinin in several non-nodulated soybean lines, although an electrophoretic protein band due to this protein was clearly detected in the corresponding nodulated isolines [47]. The suppression of the β−subunit in the non-nodulating isoline T201 is regulated at the level of mRNA accumulation. The α'−and α−subunits mRNAs were actively expressed in both line. Nitrogen regulation for storage protein in soybean seeds was evaluated using T201 and T202 with solution culture in the greenhouse [48]. The results indicated that non-nodulated T201 has a normal, non-defective, β−subunit genes, and that limited N availability decreases the accumulation of β−conglycinin.

4. Inoculation of efficient strains of bradyrhizobia to improve soybean seed yield

4.1. Inoculation of bradyrhizobia and indigenous strains in fields

Soybean plants form root nodules by symbiosis with soil bacteria *Bradyrhizobium* (*B. japonicum*, *B. elkani* and *B. lianingense*), *Sinorhizobium* (*S. fredii*, *S. xinjiangense*) and Mesorhizobi-

um (*M. tianshanense*) [49]. Only *B. japonicum, B.* elkani, and *S. fredii* are used as commercial inoculants for soybean with *B. japonicum* being the most widely employed [49]. Genus *Bradyrhizobium* belongs to α-Proteobacteria, family Bradyrhizobiaceae. *Bradyrhizobium* species are rod shaped Gram negative bacteria with a single subpolar or polar flagellum. They can be isolated from nodules and grown on a Yeast Extract Mannitol agar plate (Figure 20) [50]. Rhizobia and bradyrhizobia can exist either as a free living organism in soil or a symbiotic state in the infected cells of root nodules. When bradyrhizobia inhabit a soil, they do not fix N_2 and they depend on organic nitrogen. After they infect soybean roots and form root nodules, they become "bacteroids" in a symbiotic state and fix N_2.
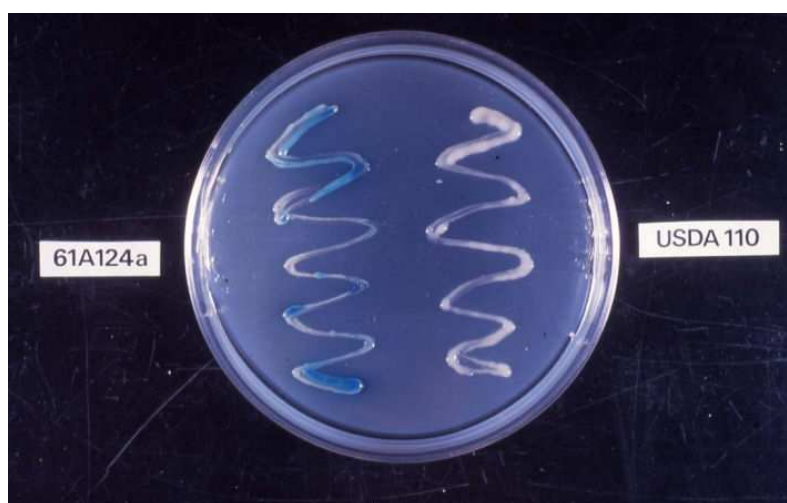

Figure 20. A culture of *Bradyrhizobium japonicum* USDA110 (right) and the *gus* mutant line 61A124a (left) on a Yeast Extract Mannitol agar plate which contain GUS substrate X-Gluc.

When soybean plants are cultivated in a new field where soybeans are cultivated for the first time, the inoculation of compatible strains of bradyrhizobia may significantly promote plant growth and seed yield. However, after cultivation of soybean, rhizobia will predominate throughout the soil. Therefore, soybean plants are usually nodulated with indigenous rhizobia in most fields of Japan without inoculation. Tewari et al. reported that the inoculation of the efficient strain of bradyrhizobia USDA110 in a paper pot filled with vermiculite was very effective in the first year of a soybean crop after 30 cm layer of mountain soil was dressed, where population of bradyrhizobia was very low [51]. Figure 21 shows the plant with uninoculated paper pot (left) and that with inoculated paper pot (right) cultivated in the mountain soil. The average seed weight per plant was 8.7 g in uninoculated plants and 22.3 g in inoculated plants, respectively. In a rotated paddy field in Nagakura where indigenous rhizobia had been established, Tewari et al. inoculated USDA110 in a paper pot, and the plant growth and seed yield was about 10-20% higher than the uninoculated paper pot (Figure 22). Good nodulation occurred in the roots of the plant in the uninoculated paper pot, because indigenous bradyrhizobia already inhabited the field.

Figure 21. The effect of inoculation of *Bradyrhizobium japonicum* USDA110 on soybean growth cultivated in montain soil where the population of indigenous bradyrhizobia was very low. Left plant was cultivated with a paper pot without inoculation. Right plant was cultivated with a paper pot with inoculation of bradyrhizobia [51].
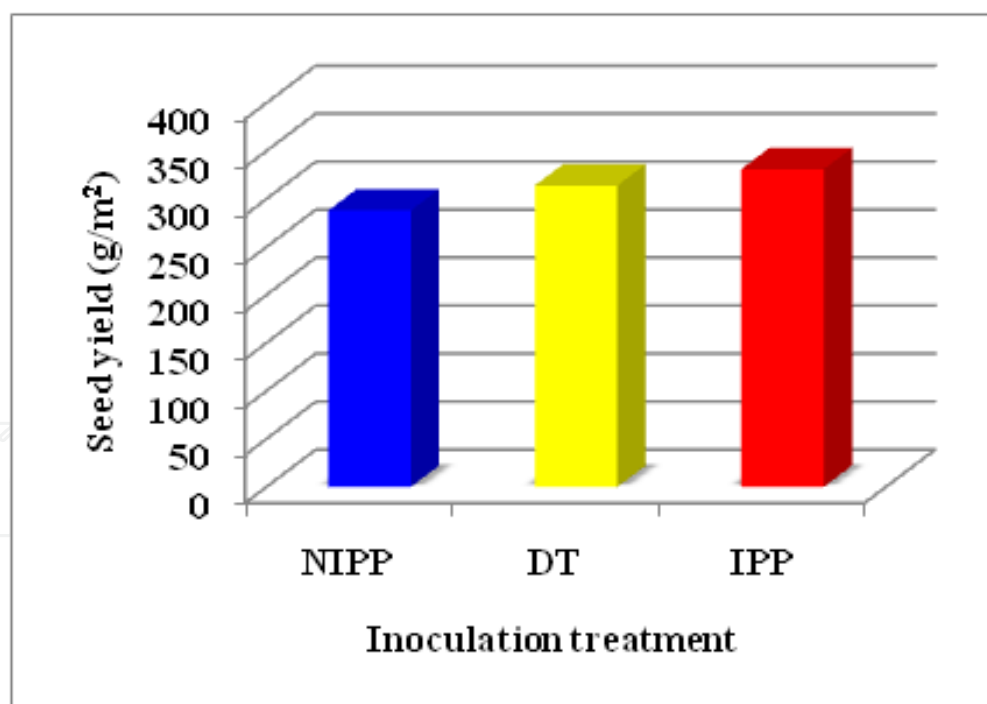
Figure 22. The effect of inoculation method for seed yield of soybean plants in a rotated paddy field in Nagakura. NIPP; Non-inocultated paper pot.DT; Direct inoculation of bradyrhizobia to soybean seed. IPP; Inoculated paper pot [52].

Because the soybean plant can form nodules in most of the fields including a rotated paddy rice field in Japan, farmers generally do not inoculate bradyrhizobia, except in the Tokachi

area in Hokkaido. The Tokachi Federation of Agricultural Cooperatives in Hokkaido provides biofertilizer inoculants for soybean seeds [50]. Although many types of bradyrhizobia exist in a field [53,54], not all are efficient strains. Minamisawa et al. reported that 44 isolates of *Bradyrhizobium japonicum* from Nakazawa field in Niigata were divided into 33 genetically different groups by genetic analysis using a repeated sequence specific hybridization method. The similar diversity of indigenous bradyrhizobia was also shown in 6 sites in Japan, including the Nakazawa and Nagakura fields in Niigata [54]. The Nakazawa and Nagakura fields are very near to one another, but bradyrhizobia types differ between the fields. In Nagakura, *B. japonicum* hup+ (uptake hydrogenase positive) and hup- (uptake hydrogenase negative) groups made up about 80% and 20% of bradyrhizobium, respectively. On the other hand, in the Nakazawa field, *B. japonicum* hup+, hup- and *B. elkanii* made up about 50%, 20% and 30%, respectively, of the local bradyrhizobium. Significant diversities and site-dependent variations were observed, and the fingerprints at Ishigaki island with no history of soybean cultivation were less diverse than the other sites. They suggested that soybean bradyrhizobia might be diversified in individual fields by association with host plants and local soil conditions [54].

4.2. Use of marker strain as an inoculant

The ecological study of inoculated strains in soil is important to establish the efficient way of inoculation. Minagawa et al. used *gus* (β-glucuronidase gene)-marked *Bradyrhizobium* strain to estimate the number of inoculated strains in the soil. The *gus* gene from *Eschrichia coli* was introduced into *Bradyrhizobium japonicum*. This strain absorbs and hydrolyzes GUS(β-glucuronidase)-substrate (X-Gluc; 5-bromo-4-chloro-3-indolyl-β-glucuronide) and precipitates an indigo pigment in the cell (Figure 20). The accumulated indigo blue metabolite in *gus*-marked strain can be determined for the population of liquid cultured rhizobia using optical density at 645 nm. However, when the *gus*-marked strain was inoculated in soil, the stained bradyrhizobia were difficult to separate from the soil. Therefore, we extracted the blue pigment by phenol-water after they are incubated with X-Gluc for 4 days. The absorbance of the extracted blue pigment in the phenol-layer was measured optically at 645 nm. The initial number of bradyrhizobia in culture media or soil and the absorbance of the phenol extract of GUS metabolite was positively correlated. In addition, the occupancy by *gus*-strain in each nodule can be determined by staining the nodule slice with substrate X-Gluc (Figure 23). The nodule occupancy by *gus*-marked strain was 50% when the same population of *gus*-marked strain and USDA110 were inoculated at the same time [55].
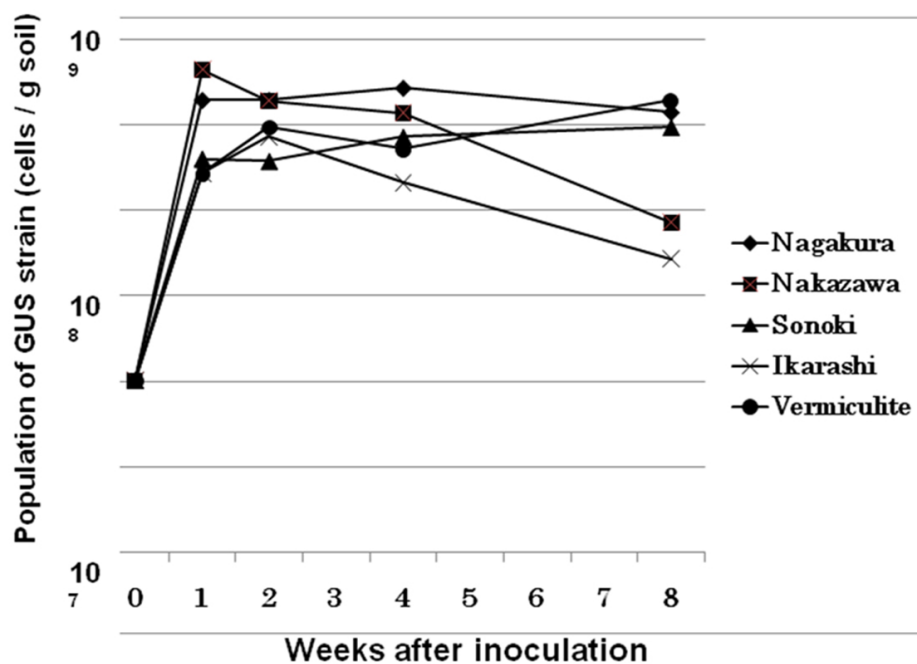
The *gus*-marked strains were inoculated in five different types of soil (Nagakura and Sonoki; two types of Alluvial soil from rotated paddy fields), volcanic ash soil of upland field (Nakazawa), sandy dune soil (Ikarashi), and calcinic vermiculite. Soybean cultivation was frequent in Nagakura and Nakazawa fields, but soybean plants have not been grown for a long time in Sonoki and Ikarashi fields. The number of indigenous bradyrhizobia was estimated by MPN method, and the populations were as follows; Nagakura (6×10^5 cells g^{-1} soil), Nakazawa (3×10^5), Sonoki (2×10^4), Ikarashi (8), and Vermiculite (0), respectively. The population of *gus*-marked strains increased over 10 times in all types of soils for 1 week after

inoculation of 9 x10^6 cells g^{-1} soil (Figure 24). The population was higher in Nakazawa and Nagakura than that in Sonoki, Ikarashi, and vermiculite at 1 week after inoculation. From this result it was indicated that the population of indigenous bradyrhizobia may not restrict the growth of inoculated strains.
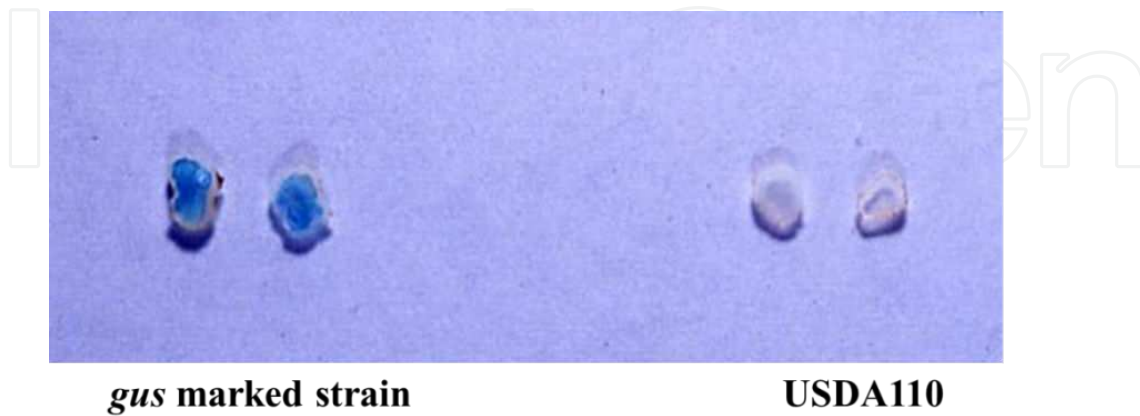
Figure 23. Detection of *gus*-marked strain in the nodule by X-Gluc treatment.

The population of inoculated strains was determined in rhizosphere soil, non-rhizosphere soil and roots (Figure 25) [51]. The population increased about 10 times in a week both in rhizosphere and non-rhizosphere soils at one week after inoculation.

Figure 24. Changes in population of inoculated *gus*-marked strain in various types of soils in Niigata.

The proliferation and mobility of inoculated *gus*-marked strains were examined in a rhizobox containing various soil types (Figure 26), where a soybean plant was cultivated in the center of the box. Inoculation and watering were supplied from one side of the box. The proliferation rates of the *gus*-marked strain in a whole box at 25 days after planting were different in various types of soils; Nagakura (x 1,218), Nakazawa (x 538), Sonoki (x 513), Ikarashi, (x173) and vermiculite (x 98). In all the soil types, bradyrhizobia were distributed in many compartments of the rhizobox (Figure 27). It was observed that most of bradyrhizobia attached to the soil particle, however, some of them moved by water flow through soil apertures or along with the root elongation.
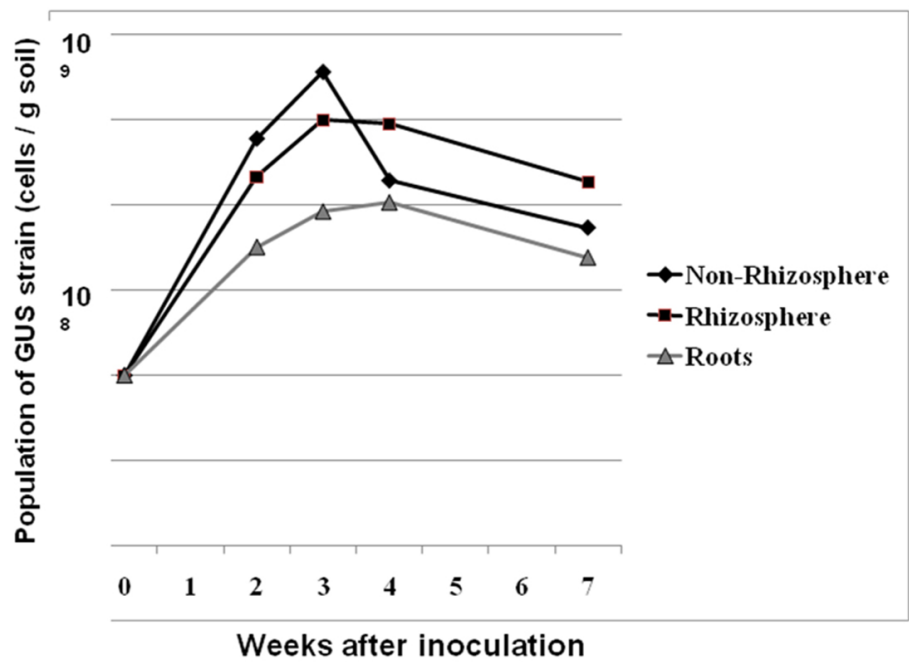

Figure 25. Changes in population of inoculated *gus*-marked strains in non-rhizosphere soil, rhizosphere soil and roots.
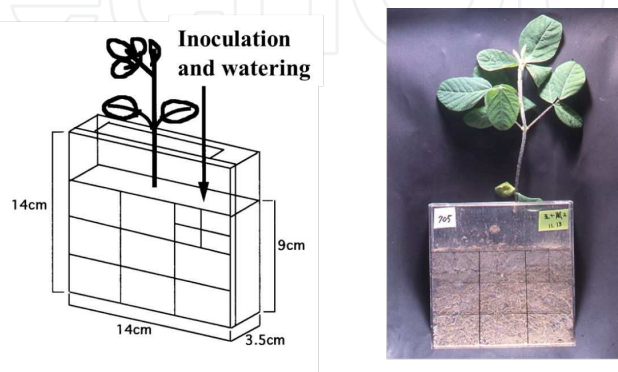

Figure 26. Rhizobox experiment system.

A soybean plant was grown in the center of the box, and inoculation of the *gus*-marked strain and the watering was carried out from one side of the box (arrow).

For soils lacking indigenous bradyrhizobia (vermiculite) or having a very low density of indigenous *Bradyrhizobia* (Sonoki and Ikarashi), the nodules were formed almost exclusively by the inoculated *gus*-marked strain Sonoki (100%; occupancy rate by *gus*-strain), Ikarashi (98%) and vermiculite (100%). However, the major nodules were formed by indigenous strains in the soil types, which contain a high population of indigenous *Bradhrhizobia*, in Nagakura (25% occupancy rate by *gus*-marked strain), Nakazawa (35%*gus*-marked strain), although the inoculated strain proliferated very well. The results suggested that nodule occupancy may be simply related to the population rate of inoculated strains vs. indigenous strains rather than the competition between inoculated and indigenous strains.
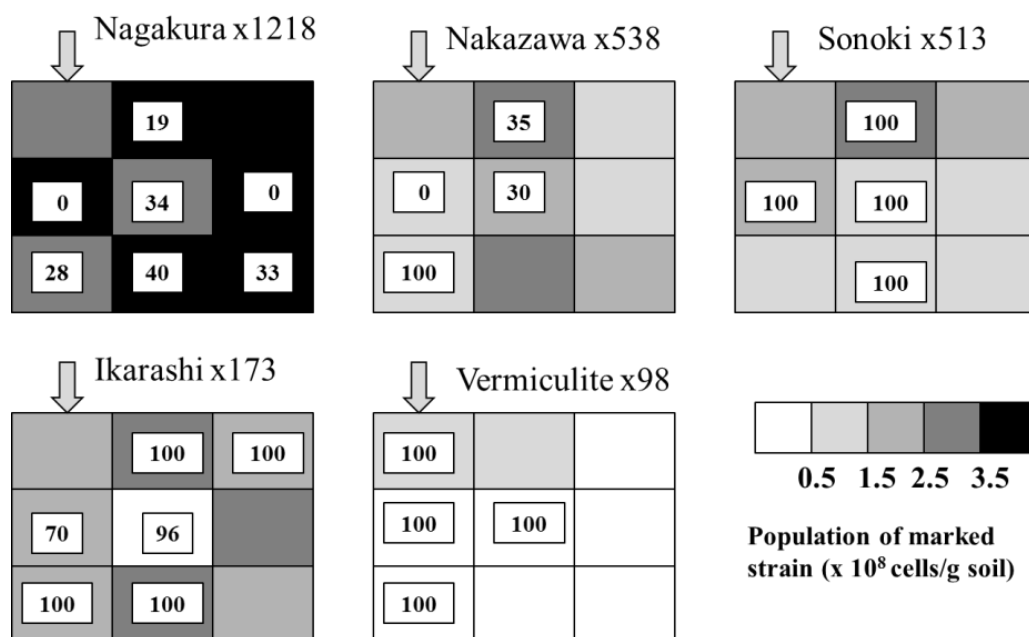

Figure 27. Population of *gus*-marked strains in 9 compartments of each rhizobox (color of each compartment), and the percentage of nodule occupancy by *gus*-marked strains (number in the center of each compartment) [55]. Arrow indicates the site of inoculation and watering.

4.3. Survival of inoculated *gus*-marked strain

The survival of the inoculated strain is very important to improve indigenous strains in a field. We investigated the survival of *gus*-strains in the first soybean cultivation just after inoculation of the *gus*-strain, and in the second year in the pot filled with five soil types. The nodule occupancy rate (%) by the *gus*-marked strain in the first and second year are shown in Figure 28. In the Ikarashi and vermiculite soils with low densities of indigenous bradyrhizobia, the nodule occupancy rates by the *gus*-marked strains were high (about 80-100%) both in the first and second year. However, in Nagakura, Nakazawa and Sonoki soils, the nodule occupancy rates were lower in the second year than the first year. This result sug-

gests that the survival ability of the inoculated single strain may be inferior to the indigenous strains. The genetic diversity of the indigenous strain may be related to the competition between inoculated and indigenous strains.
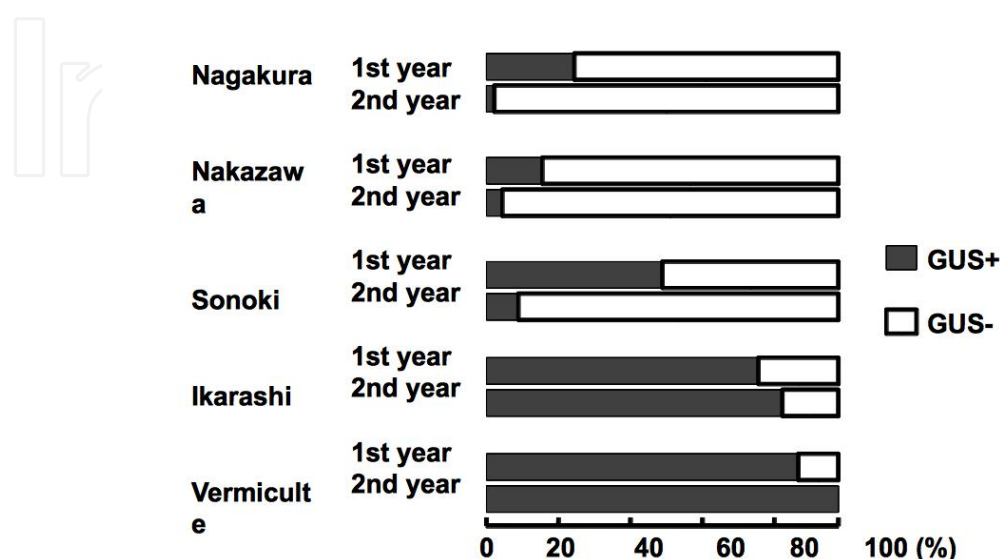
Figure 28. Percentage of nodules occupied by *gus*-marked strain (GUS+) and indigenous strain (GUS-) in various soils in Niigata in the first and second cropping years after inoculation.

5. Field estimation of nitrogen fixation activity and nitrogen absorption rate of soybean by relative ureide method

5.1. Concept of relative ureide method

Many tropical grain legumes, such as soybean, common bean, cowpea, pigeon pea, and mung bean that have the spherical determinate type of nodule, transport the bulk of fixed N as ureides (allantoin and allantoic acid). On the other hand, nitrate and amino acids (especially amide, asparagine) are the major transport forms of N derived from the soil and fertilizer N absorbed by soybean roots [36, 56]. Herridge et al. [57,58] developed the "Relative ureide method" for evaluation of % Ndfa by analyzing the concentration of nitrogen compounds in xylem sap obtained from the bleeding sap from a cut stump, or vacuum collection from stems. The concentrations of ureide-N, nitrate-N and α-amino-N can be determined by colorimetry.

Figure 29 shows a concept of relative ureide mathod for estimation of nitrogen fixation activity and Figure 30 shows the equation of %Ndfa by this method. The xylem sap obtained

from soybean plants, which depend solely on nitrogen fixation contains about 80-90% of N in the form ureides (allanotate and allantoin), in addition to some amino acids such as asparagine. On the other hand, in the xylem sap from soybean plants depending only on nitrate, nitrate and amino acids (asparagine) are the major N compounds accounting for 80-90%, and they contain about 10-20% as ureides. When the xylem sap in N_2 grown soybean contains about 10-20% of N as amino acids, and nitrate grown soybean contains the same percentage of N as ureides, the relative dependence on N_2 fixation is estimated by the relative ureide content as shown in Figure 29.
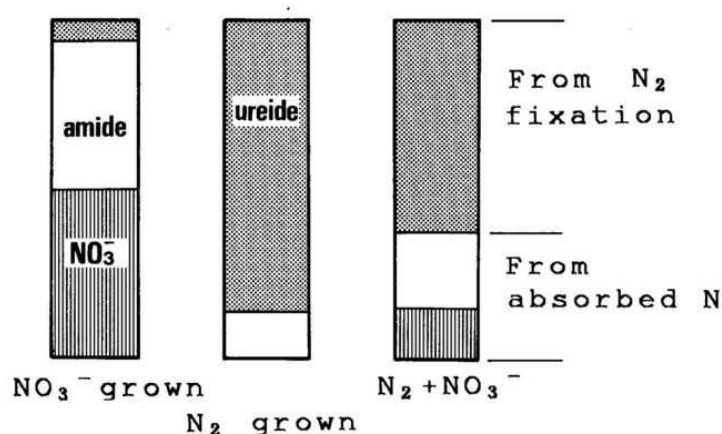

Figure 29. Concept of the relative ureide method using the concentration of xylem solute ureide-N, nitrate-N and amino-N in soybean. NO_3^- grown; Plants depend on sole NO_3^- absorption. N_2 grown; Soybean plants depend on sole N_2 fixation, $N_2 + NO_3^-$; Soybean plants depend on both N_2 fixation and NO_3^- absorption. [16,50]

This method is reliable in soybean field experiments, without any requirement of reference plants. It is the easiest way to measure the percentage of fixed N in any fields, because no preparation is necessary before sampling. This method is also applicable for experiments with variable N fertilizer application. In field conditions, the simple equation can be adapted for the estimation of % Ndfa (nitrogen derived from atmospheric dinitrogen) by the equation in Figure 30. The original equation proposed by Herridge used "α-Amino-N" instead of "2 X α-Amino-N" in our equation. Based on the analysis of the animo acid compositions in root bleeding xylem sap, asparagine (2N amide) was the major amino acid throughout the stages and the average N number in amino acids was 1.7, so we use "2 X α-amino-N" for this equation.

$$\%Ndfa = \frac{Ureide\text{-}N}{Ureide\text{-}N + Nitrate\text{-}N + 2 \times \alpha\text{-amino-N}}$$

Figure 30. Equation for calculating percentage of nitrogen derived from atmospheric N_2 (%Ndfa).
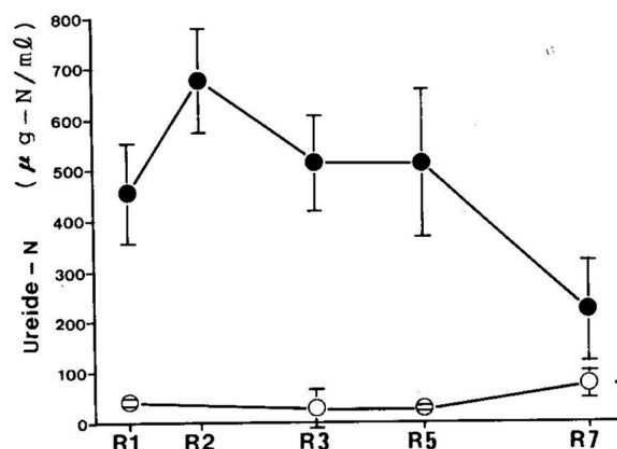
Figure 31. Changes in the concenration of ureide-N in nodulated T202 (closed circle) and non-nodulated T201 (open circle) [16].
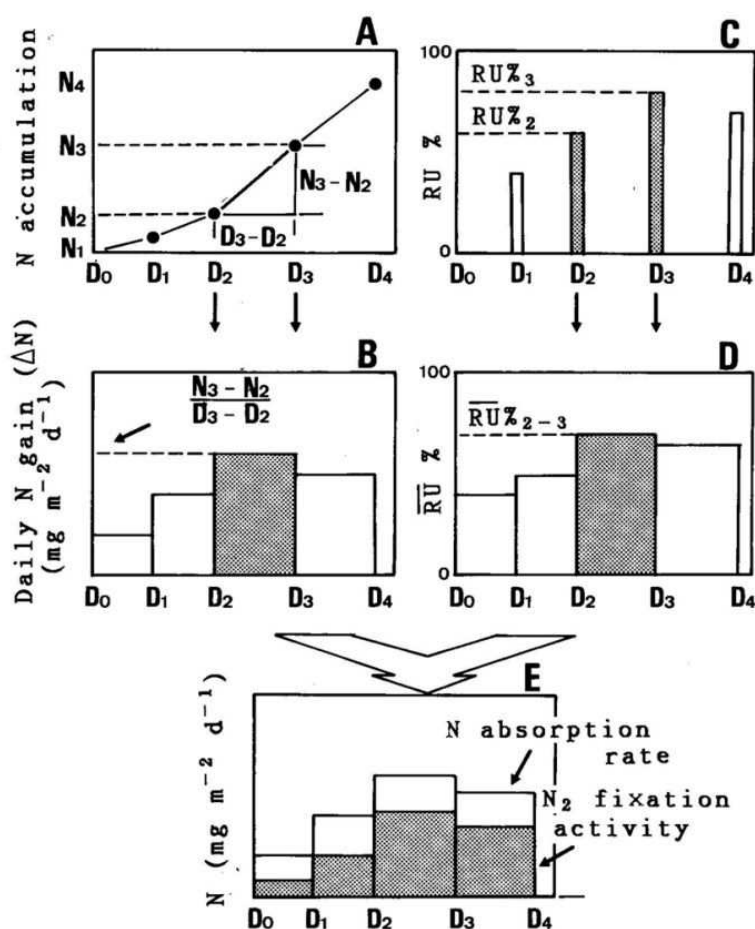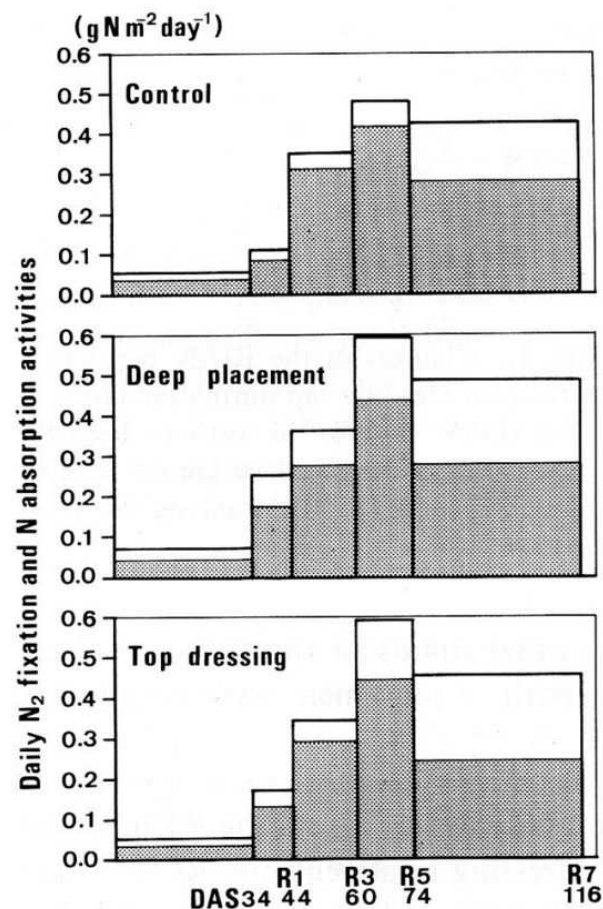

Figure 32. Outline of estimation of daily N_2 fixation activity and N absorption rate based on the data obtained by relative ureide-N and total N analysis of the shoot [16,50].

Figure 31 shows the changes in the concentration of ureide-N in xylem sap of nodulated (T202) and non-nodulated (T201) soybean plants [16]. The concentration of ureide-N in T202 was significantly higher than that in T201. In the field estimation, the relative ureide N per-cent is used as an indicator of relative dependence for nitrogen fixation at the sampling time.

5.2. Quantitative estimation of daily N₂ fixation and N absorption rate

By periodic sampling of soybean shoots and xylem sap, a quantitative estimation of the sea-sonal changes in N_2 fixation activity and N absorption rate is possible as shown in Figure 32 [16]. We usually sample soybean plants four or three times at R1, R3, R5 and R7 stages or three times at R1, R5, and R7 stages for xylem sap and plant N analyses. The examples of the evaluation of Ndfa by relative ureide method are shown in Figure 33 [4].

Figure 33. Example of the estimation of daily N_2 fixation activity and N absorption rate based on the data obtained by relative ureide-N and total N analysis of the shoot. Three fertilizer treatments were used: control; without additional fertilizer, Deep placement; deep placement of 100 day type coated urea, Top dressing; top dressing of 70 day type coated urea [4].

D_1, D_2, D_3, D_4 indicate the sampling date (Days after planting) of xylem sap and shoots. We usually sample at R1, R3, R5 and R7 stage for D_1, D_2, D_3, D_4. D_0 means the planting date. RU %$_n$ indicates the relative ureide N percent in xylem sap at sampling time at D_n. RU%$_{n-n+1}$ means average of RU% at D_n and D_{n+1}. We use RU%$_{1-0}$ as RU%$_1$, because we cannot measure the RU% at planting.

6. Possiblity of the agronomical use of hypernodulation mutant lines of soybean for promoting yield

Soybean plants can control the nodule number by auto-regulation of nodulation. Supernodulation or hypernodulation mutant lines of soybean have been isolated from wild type soybean, and it was expected that these mutants would produce higher seed yield. However, most of these mutants showed reduced vegetative growth and seed yield, possibly due to a heavy burden of excess nodules. Hypernodulation or supernodulation mutant lines of soybeans have been selected from several different cultivars [59-61]. A genetic defect in the autoregulatory control of nodulation causes more profuse nodulation than the wild type (Figure 34). The nodulation trait depends on the shoot genotype and not on the root genotype. In the wild type parents, some shoot-derived signal (autoregulation signal) arrests nodule primordia and suppresses nodule development in response to some signals (infection signal) derived from nodulated roots after infection. Sato et al. reported that a rooted single leaf of soybean retains the autoregulation trait.

Recently, the genes were identified for the hypernodulation lines of lotus (*HAR1*) and soybean (*GmNARK*), which play important roles in the autoregulation of nodulation, and they were shown to encode a receptor-like kinase protein that contains a leucine-rich repeat [64-66]. These legume genes are homologous to *Arabidopsis CLAVATA1* (*CLV1*), which is involved in the control of cell proliferation in the shoot apical meristem [67]. The results by Ito et al. suggest that the protein coded by GmNARK may play some roles on leaf growth as well.

In spite of profuse nodulation in the hyprnodulation mutant lines, the root and shoot growth is inferior in most of these lines compared with the wild type with or without nitrate supply. All the hypernodulation mutant lines are partially tolerant to NO_3^-. The supernodulation line first reported was nominated "nts", which means "nitrate-tolerant symbiosis" mutant [60]. The labeling experiments using $^{14}CO_2$ or $^{13}CO_2$ indicated that the hypernodulating mutant NOD1-3 supplied a larger amount of photoassimilate to the nodules than to the roots under nitrogen free conditions, and that the photoassimilate transport to the nodules was less sensitive to nitrate than that of the parent line [69].

Assimilation of $^{15}N_2$ and $^{15}NO_3^-$ was compared among hypernodulation mutant lines, NOD1-3, NOD2-4, and NOD3-7 and the parent Williams [70]. The 5 mM NO_3^- treatment resulted in a 95 to 97% decrease in nodule mass and $^{15}N_2$ fixation by Williams, while the three mutant lines retained 30 to 40% of the nodule mass and 17 to 19% of the $^{15}N_2$ fixation of control Williams. The hypernodulation mutant lines, which had restricted root growth, absorbed less $^{15}NO_3^-$ than Williams. These results confirmed that nodule formation and

development are less sensitive to external NO_3^- in mutant lines than in the Williams parent. The partial tolerance of nodulation for nitrate in mutant lines may be partly due to less NO_3^- absorption activity and smaller roots.

Figure 34. Nodulated roots of Williams (left) and the hypernodulation mutant NOD1-3 (right).
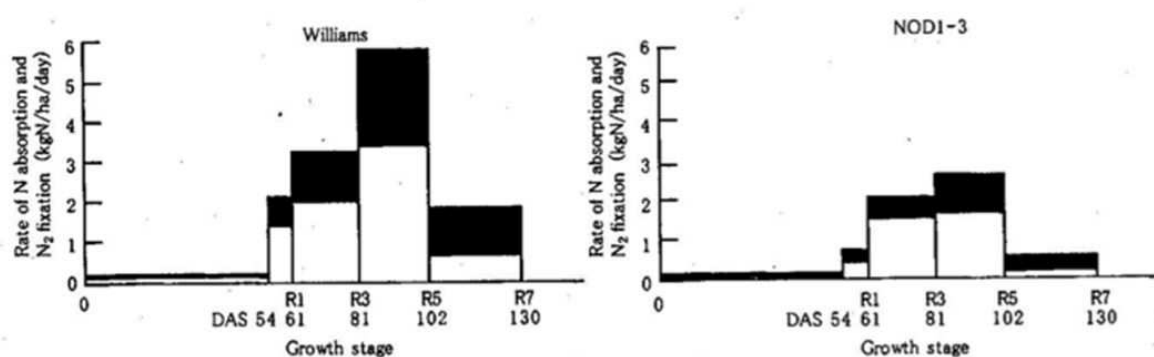
Figure 35. Changes in daily N_2 fixation activity and N absorption rate by Williams and the hypernodulation line NOD1-3 cultivated in a sandy dune field in Ikarashi [6].

The hypernodulation mutant lines of soybean may have some advantages for promoting seed yield, due to higher N_2 fixation activity or the nitrate tolerant trait to nodulation. Wu

and Harper evaluated the N_2 fixation potential and yield of hypernodulating soybean NOD1-3, NOD2-4 and NOD3-7 compared with the parent Williams. In the absence of N fertilizer, all hypernodulation mutants had greater N_2 fixation potential than did Williams in early growth stages. However, the seed yields from the hypernodulation mutants were 10 to 30% less than that for Williams. Suganuma et al. also compared the growth and N_2 fixation activity of NOD1-3 and Williams in a sandy dune field. Figure 35 shows the daily rate of N_2 fixation and N absorption by Williams and the hypernodulation mutant NOD1-3. The % Ndfa was higher in NOD1-3 (65 %) than Williams (58 %), however, the rates of N_2 fixation and N absorption were lower in NOD1-3 than in Williams. The hypernodulation mutant lines have not been used for cultivar improvement, but recently "Sakukei no. 4" bred from En6500 (hypernodulating line from "Enrei") and "Tamahomare" in Japan may be useful in agricultural production by increasing the planting density.

7. A new fertilization technique to promote nitrogen fixation and seed yield

7.1. Nitrogen fertilization in soybean cultivation

Nitrogen fertilization usually depresses nodulation and nitrogen fixation activity. It often results in the same or less yield than the control treatment without fertilizer. Top dressing of N fertilizer sometimes gives positive effects on seed yield, but results are not consistent. Takahashi et al. reported the effect of basal side-dressings of various types of controlled release nitrogen fertilizer (coated urea) on shoot growth and seed yield of soybean [73]. The yield was significantly higher in all the coated urea basal side-dressing treatments compared with control, particularly in CUS120 which releases urea from 60 to 120 days after planting with a sigmoid pattern of N release.

We have developed a new fertilization technique for soybean cultivation to supplement N during seed filling stage, without the concomitant depression of N_2 fixation, by deep placement (20 cm depth from soil surface) of slow release N fertilizer coated urea and lime nitrogen. We analyzed the beneficial effects from both plant nutrition and soil analysis aspects.

7.2. Deep placement of controlled release nitrogen fertilizer coated urea

Takahashi et al. [4,16, 74-77] developed a new fertilization technique for soybean to supplement nitrogen during the seed filling stage without concomitant depression of symbiotic N_2 fixation by deep placement of coated urea, a slow release N fertilizer. They applied 100 kg N ha^{-1} coated urea by deep placement (20 cm depth from soil surface) using a fertilizer injector devised by Shioya [78]. They used CU-100, a 100-day type coated urea, the commercial name "LP-100" produced by Chisso-Asahi Fertilizer Co. Ltd, Tokyo (JCAM AGRI Co. Ltd., Tokyo at present). CU-100 linearly releases urea and 80 % of which is released in 100 days in water at 25°C.

A polymer coated controlled release nitrogen fertilizer (commercial name LP in Japan or MEIS-TER outside Japan) has been invented by Fujita and coworkers [79]. Linear types of coated urea were first marketed in 1982. This type of fertilizer has a spherical shape of about 3mm diameter with 50-60 μm coat thickness which consists of polyolefin (polyethylene), ethylene vinyl acetate and talc mineral. Different from chemically synthesized slow release N fertilizers such as IBDU (Isobutylidene diurea) and CDU (Crotonylidene diurea), the N release rate from the coated urea is temperature dependent and not affected by other chemical, physical and biological conditions. Therefore, the release pattern of urea can be predicted as a function of temperature and time period after application. Since the release of N from the fertilizer meets the plant N demand, and the fertilizer efficiency (recovery rate of N in plants from fertilizer N) is high, the use of coated urea can reduce environmental problems by decreasing nitrate accumulation and leaching in the soil. Also the use of coated urea saves the labor of farmers by eliminating top dressing or split dressing of fertilizer to supply N during late growth stages.

Fertilizer experiments were carried out from 1989 to 1991 in the fields, which had been converted from a paddy rice field the previous year in Nagakura. [21]. As shown in Figure 36 the seed yield was significantly higher in the plants with the deep placement of CU-100 than the control in each year. The seed yield was from 10 (1991) to 23 % (1990) higher in deep placement than the control treatments. The promotion of leaf growth and retardation of leaf senescence were observed at the maturing stage by deep placement of CU. In 1990, the seed yield was very high, about 6 t ha^{-1} in deep placement, due to favorable climatic conditions. The absorption efficiency of fertilizer N determined by ^{15}N labeled fertilizers was calculated from recovery of ^{15}N in the shoots at the R7 stage. In 1990, the absorption efficiency at R7 from the deep placement of CU-100 was 62 %, which was much higher than the top dressing of CU-70 (33 %) and basal application of ammonium sulfate (9 %). It was observed that the CU-100 deep placement increased root growth and water and nutrient absorption activity revealed by the uptake of rubidium tracer in the field [75]. Owing to the promotion of subsoil root growth and N absorption activity with supplementing N fertilizer without depression of N$_2$ fixation, plant growth was promoted from the early vegetative stage until late maturing. Leaf area index (LAI) and chlorophyll content were always higher in CU-100 deep placement compared with the control, and the leaf senescence was retarded at R7 stage [21].

Takahashi et al. analyzed the concentration of urea, ammonium and nitrate in the upper 0-10cm and lower 15-25cm layers of the soil in control and deep placement of CU-100 treatments. In the upper layer, the concentration of urea and nitrate was very low (less than 10 mg N kg^{-1} soil) both in control and deep placement of CU-100 treatments. However, the accumulation of ammonium (up to 150 mg N kg^{-1}) and nitrate (up to 50 mg N kg^{-1}) was observed in the lower layer of deep placement of CU100 in August. Although the urea released from the coated urea was rapidly hydrolyzed to ammonia, NH$_4^+$-N could not be easily nitrified in the deep soil layers of the converted rice field owing to the low activity of nitrification and restricted O$_2$ supply. As a result, the nodulation and N$_2$ fixation near the surface layer were not depressed, and instead were promoted through the improvement of plant growth and photosynthetic activity. The mechanism of promotion of deep placement of coated urea for soybean growth and seed yield is summarized in Figure 37.
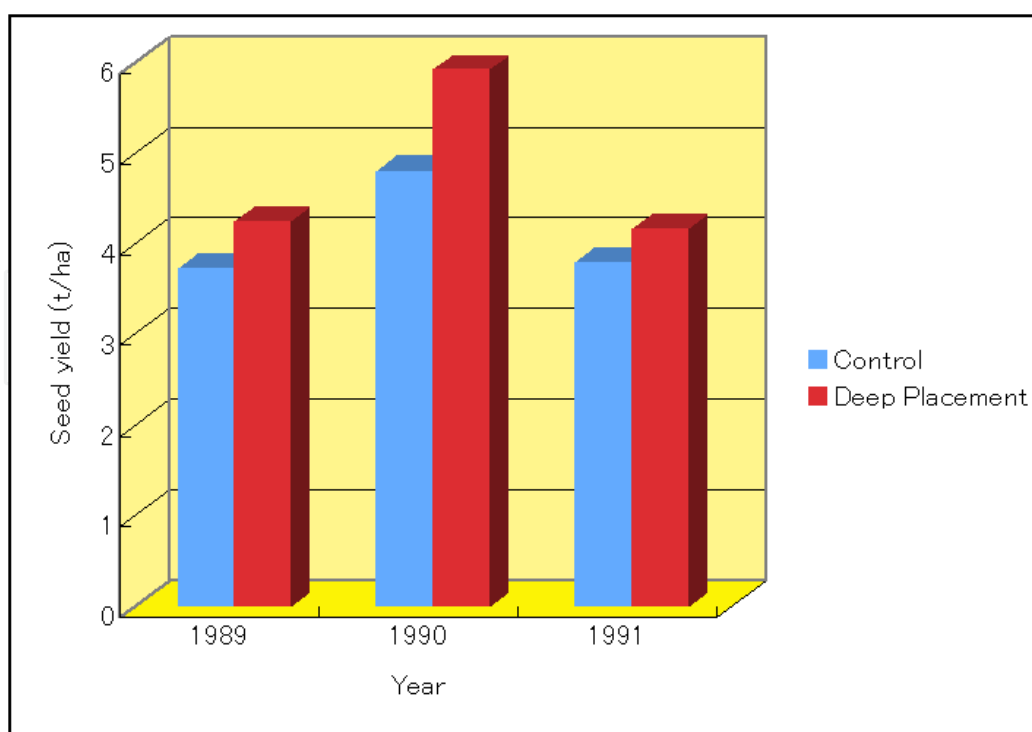
Figure 36. Comparison of the seed yield of control and deep placement of coated urea in rotated paddy fields in Nagakura in 1989, 1990 and 1991. [4]
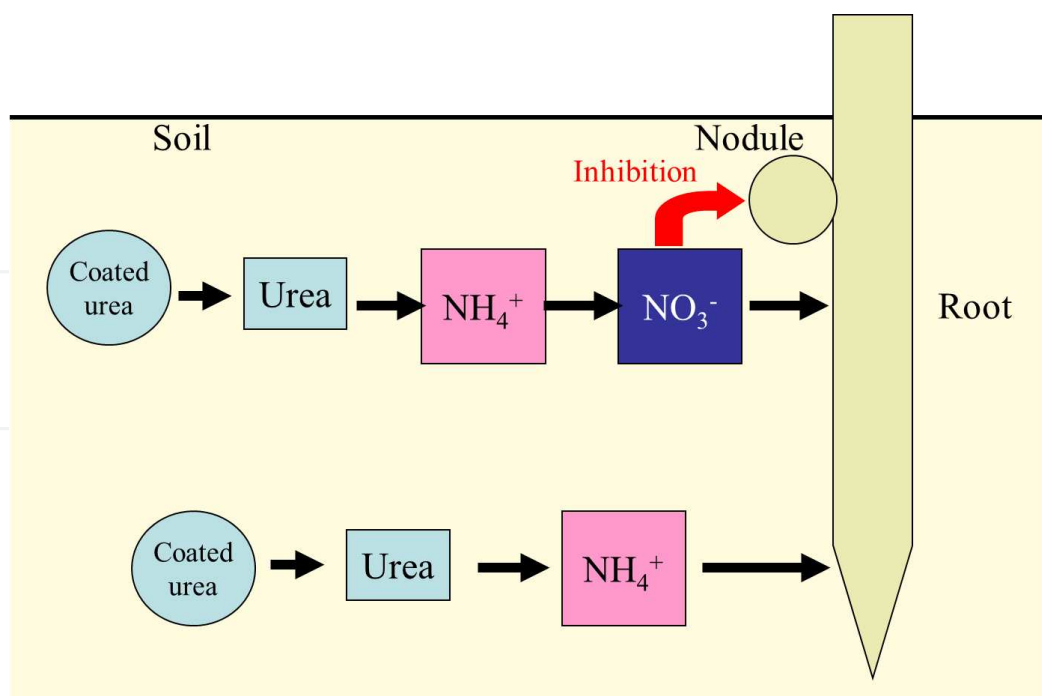
Figure 37. A model of the promotive effect of deep placement of coated urea on nitrogen fixation and seed yield of soybean.

a. Deep placement of coated urea slowly releases urea inside. The urea is rapidly hydrolyzed to ammonium and the ammonium does not leach out from the fertilization sites at 20cm depth.

b. The abundant supply of N in the deep layer promotes the root growth, and water and nutrients absorption activity and fertilizer N is efficiently absorbed from the lower roots.

c. The abundant supply of N from the lower part of roots promotes leaf growth and extends the photosynthetic activity until maturity. The leaf area and chlorophyll content were higher in the leaves of deep placement than those in control ones.

d. An abundant supply of photoassimilate to nodules supports the nodule growth and N_2 fixation activity for an extended period during the seed filling stage.

e. The continuous supply of N from nodules and roots with increased photo assimilate supply promotes seed yield with good quality.
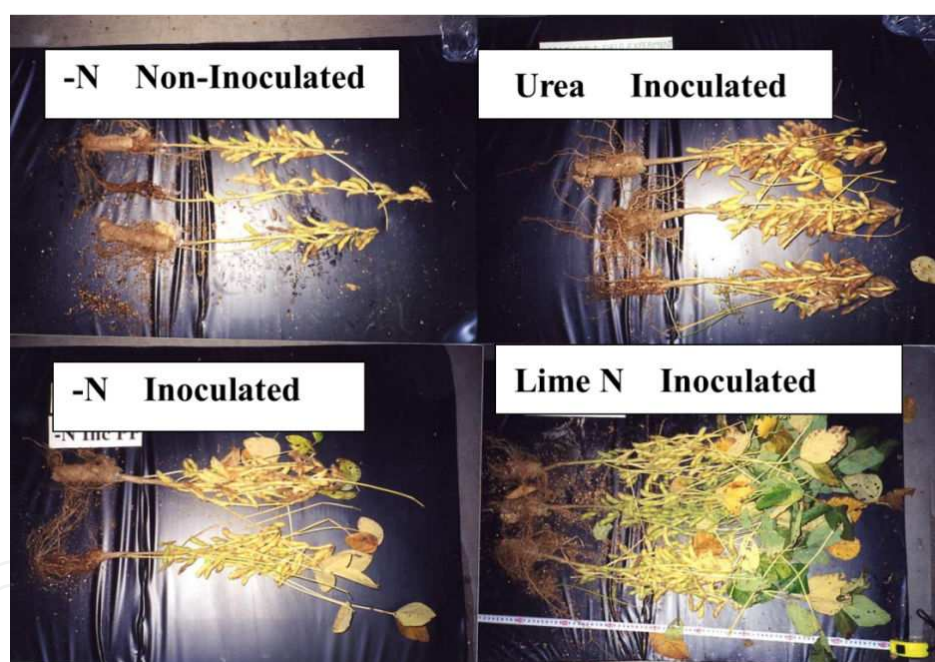
Figure 38. Soybean plants at R7 cultivated in a newly reclaimed field with a 30 cm depth of mountain soil. Plants were planted with inoculated or non-inoculated paper pot. –N: without N deep placement, Urea: deep placement of urea at 20cm depth. Lime N: deep placement of lime nitrogen. [51]

7.3. Deep placement of lime nitrogen

Recently, Tewari et al. [51,52,80,81] investigated the effects of deep placement of lime nitrogen (calcium cyanamide, $CaCN_2$) in comparison with coated urea. The fertilizer experiments were combined with a new inoculation method of bradyrhizobia using a paper pot transplantation. All the experiments were carried out in 2001 at three different sites in Niigata

Prefecture. The tests were initiated in a field reclaimed with the application of mountain soil about 30 cm depth without indigenous bradyrhizobia (Figure 38) [51], a rotated paddy field in Nagakura in Niigata Agricultural Research Institute (Figure 39) [52], and a sandy dune field of Niigata University in Ikarashi [80].
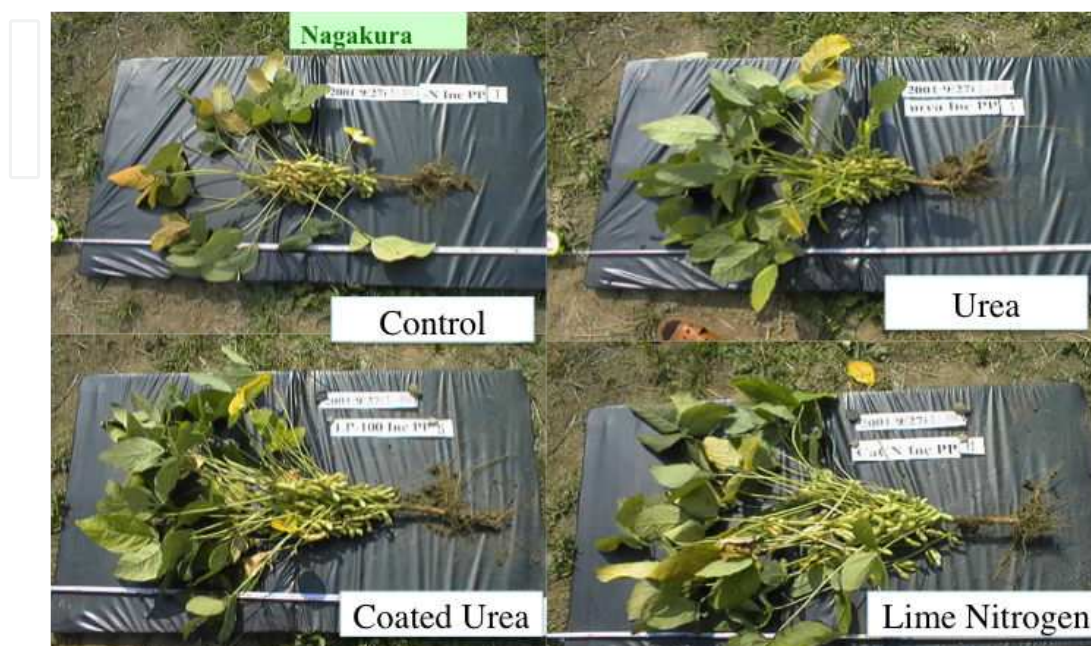
Figure 39. Soybean plants cultivated in a rotated paddy field in Nagakura. Plants were planted with inoculated paper pots. Control: without N deep placement, Urea: deep placement of urea at 20cm. Coated Urea: deep placement of coated urea. Lime Nitrogen: deep placement of lime nitrogen. [52]

Lime nitrogen is composed of about 60% calcium cyanamide ($CaCN_2$) with calcium oxide and carbon, and the N content is about 20-23%. After application to the soil, the calcium cyanamide is converted to urea, which is again degraded into NH_3 and CO_2. Dicyandiamide contained in lime nitrogen or formed during the degradation of calcium cyanamide is a potent nitrification inhibitor, which retards the oxidation of NH_4^+ to NO_3^-. Therefore the ammonium produced by $CaCN_2$ decomposition persists for a longer period of time and the nitrate concentration remains low in the soil. It is expected that the inhibition of nodulation and of the N_2 fixation activity may be alleviated by low a level of nitrate accumulation. Also this fertilizer exerts some hormonal effects on plants and is used for controlling soil diseases caused by bacteria and fungi.

In each of these fertilizer treatments, IPP (Inoculated paper pot), DT (Direct transplantation of inoculated seedlings without paper pot) and NIPP (Non-inoculated paper pot) seedlings were transplanted in separate plots. Paper pots (height 13.5 cm, diameter 3 cm) were made of a biodegradable paper designed to break down in the field. The pots were open at the bottom to allow root expansion below the pot. A paper pot was filled with vermiculite and a seed was planted in each pot, and followed by inoculation of one ml suspension of *Bradyrhizobium japonicum* USDA110 of about 10^8 cells ml^{-1}. Since the bradyrhizobium population in-

creases about 100 times in vermiculite for a few weeks [55], efficient infection of inoculated bradyrhizobia can also be expected by paper pot inoculation with vermiculite.

In regard to the inoculation method, IPP (inoculated paper pot) tended to show the highest seed yield than DT and NIPP treatments. Especially in the newly reclaimed field having mountain soil with no indigenous rhizobia at the 30 cm depth, the inoculation by IPP or DT promoted seed yield to a level more than twice as much as the control treatment. Among the inoculation methods, the IPP and DT seedlings showed a higher seed yield than the NIPP seedlings.

Significantly higher seed yields in the rotated paddy field were obtained with the deep placement of $CaCN_2$ IPP (6.12 t ha^{-2}) and CU-100 IPP (6.04 t ha^{-2}), compared with the Urea IPP (4.67 t ha^{-2}) and Control IPP (3.31 t ha^{-2}) treatments (Figure 39) [51]. A similar effect was observed in a reclaimed field and sandy dune field, where deep placement of lime nitrogen gave the same or better seed yields compared with coated urea. Recently, Sakashita et al. reported the promotive effect of deep placement of lime nitrogen in 8 sites of farmers' field in 2008, 2009 and 2010. Seed yields increased about 30% on average by deep placement of lime nitrogen.

The mechanism of yield promotion by deep placement of lime nitrogen for soybean growth and seed yield is summarized in Figure 40.
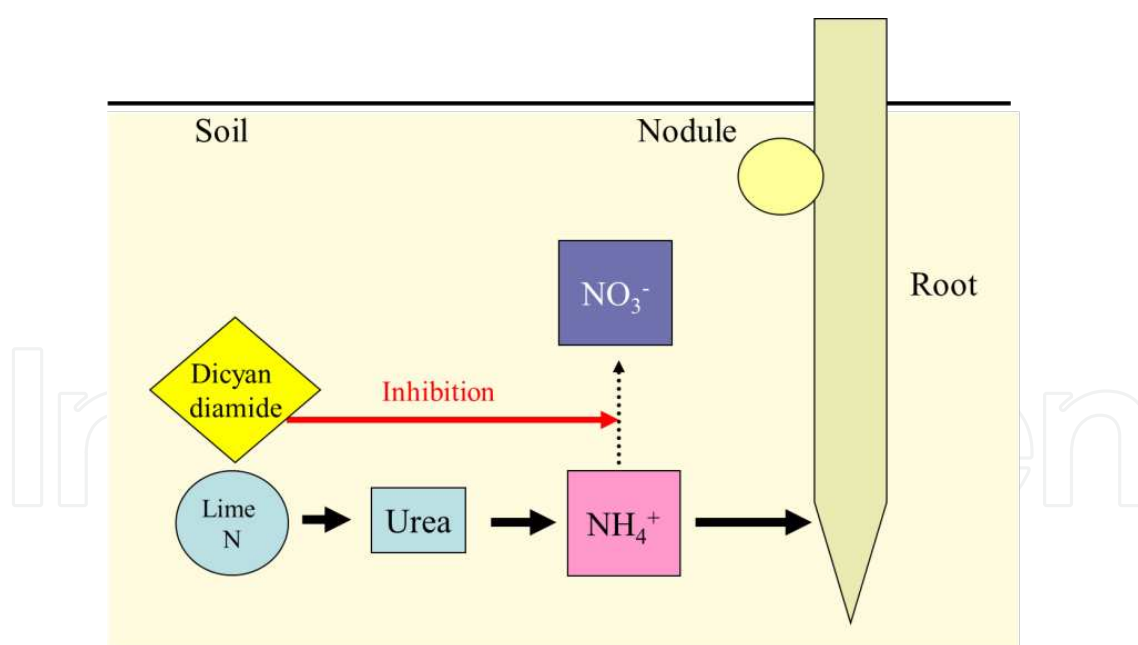
Figure 40. A model for the promotive yield effect of deep placement of lime nitrogen on nitrogen fixation and seed yield of soybean.

a. Deep placement of lime nitrogen is hydrolyzed to urea, then to ammonium and carbon dioxide. The ammonium does not easily leach out from the fertilization sites at 20 cm depth. Dicyandiamide contained in lime nitrogen or formed in the soil from cyanamide

depresses nitrification to prevent ammonium oxidation to nitrate. As a result, nitrate leaching and denitrification is reduced and the ammonium can be sustained in the sub-soil for a long time.

b. The abundant supply of N in the lower layer promotes the lower root growth, and water and nutrient absorption activity and fertilized N is efficiently absorbed from the lower roots.

c. The abundant supply of N from lower parts of roots promotes leaf growth and extends the photosynthetic activity until maturity.

d. An abundant supply of photoassimilate to nodules supports nodule growth and N_2 fixation activity for an extended period until maturity is reached.

e. The continuous supply of N from nodules and roots with increased photoassimilate supply promotes seed yield without decreasing the quality.

8. Conclusion

Potential soybean seed yield may over 10 ton ha^{-1} based on recent world record soybean production in the USA. However, the average soybean seed yield is only 2.56t. Soybean seed yield is proportional to the total assimilation of nitrogen. Soybean seeds contain an extraordinary high concentration of protein (about 35%), therefore, one t of seeds requires about 70-90 kg of N. To get a high seed yield, soybean plants need to assimilate a sufficient amount of nitrogen not only during vegetative stages but also during reproductive stages. At first, a high and long lasting activity of nodules during the pod filling stage is the most important factor to get high seed yield. To obtain optimum nodulation and nitrogen fixation activity, physical, chemical and biological conditions in the soil are very important in addition to appropriate cultivation method (fertilization, planting date, planting density, weed and pest management etc.). Inoculation of effective bradyrhizobium strains may improve soybean growth and seed yield. Although it is well known that a heavy supply of nitrogen fertilizer severely depresses nodulation and nitrogen fixation of soybean, a continuous supply of a low level of combined nitrogen from the subsoil (eg. soil organic matter) may support the plant vigor and photosynthetic activity and promote nitrogen fixation. The use of coated urea and lime nitrogen for deep placement has been successful in various types of soil in Japan.

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